Development of guidance for assessing the impact of mixtures of chemicals in the aquatic environment

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EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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EXECUTIVE SUMMARY

Activities in Europe continue to highlight the presence of chemical combinations in the environment. Furthermore, there is increasing concern about the potential impact on the environment of mixtures of chemicals and the perception that current risk assessment procedures are inadequate. The toxicity of chemical mixtures is relatively well understood through the concepts of concentration addition and independent action, with synergism being acknowledged as only a rare occurrence. It is generally accepted that concentration addition should be used as a default first tier in environmental risk assessment of mixtures. Prospective risk assessments are generally done at the level of single substances, some of which are in fact mixtures themselves, or known mixtures of substances in chemical products. The assessment factors employed in the different conservative risk assessment processes under which these are regulated may cover the potential for any combined effects from exposure to multiple substances.

The problem is that in the real world, predicting the chemicals to which an environment is exposed is difficult and often impossible. A framework is presented here which will retrospectively allow the evaluation of the potential impact of chemicals or chemical mixtures in the environment.

Initially, this involves identification of an impact which may be obvious or may be more subtle requiring the comparison of the site to a reference site. Methods to identify impacts include traditional taxonomic based indices, such as the River Invertebrate Prediction and Classification System (RIVPACS) to the trait-based methods developed more recently. These methods determine the ‘ecological status’ of a site by comparison with a reference condition.

Well established methods such as Whole Effluent Toxicity testing and Direct Toxicity Assessment have been successfully employed to indicate the potential for environmental impacts from chemicals. Toxicity Identification Evaluation and Effects Directed Analysis can subsequently be used to identify the toxic components within effluents or environmental samples allowing effective management.

Where there is no obvious cause of environmental impacts, causal analysis needs to be employed to identify probable cause as these are many, ranging from habitat, including a number of natural perturbances, to anthropogenic stressors of which toxic chemicals are one. Examples of eco-epidemiological studies from US and Europe illustrate how these studies should be conducted using various statistical and “Weight of Evidence” (WoE) methods to identify probable cause, which is necessary before employing risk management options to mitigate or remediate effects from chemicals.

1. INTRODUCTION

The potential risk from combinations of chemicals in the environment has long been a concern. However, this issue has recently moved up the scientific, regulatory and political agenda, with the realisation and demonstration that man and his environment are continually exposed to a variety of chemical compounds, not singly but in combination. This has led to concerns that there must be some impact from exposure to chemical mixtures or a ‘cocktail effect’. The natural environment is of course a mixture of chemicals, although the focus is often on man-made compounds – products of the ‘chemical industry’ – or perhaps those natural compounds which are emitted into the environment through industrial activity such as metals and mining. Indeed, the term ‘mixture’ is readily applied to the ecotoxicology and environmental risk assessment of chemicals. However, there are different kinds of mixtures to consider which can be classified into 4 broad categories:

1. Multi-constituent substances (e.g. defined reaction products such as isomeric mixtures) and UVCB substances – substances of unknown or variable composition, complex reaction products or biological materials – such as petroleum oils, natural dyes and essential oils.
2. Chemical formulations and preparations made by blending two or more different substances in specific proportions such as plant protection products, biocides, pharmaceuticals and other consumer products.
3. Mixtures of chemicals likely to occur due to the release of chemicals in the environment co-occurring in time and space, such as effluents or tank mixed plant protection products. Effluents may be relatively stable and continuous – such as refinery effluents – or fluctuating in concentration and chemical composition such as discharges from waste water treatment plants in urban areas.
4. Complex mixtures in the environment of unknown composition, consisting of anthropogenic discharges together with natural sources of chemicals.

Although all these mixtures may be defined, they share the common characteristic that in the environment their composition changes both temporally and spatially due to the different environmental behaviour of their components.

Whilst the theory of mixture toxicity has received increasing attention over recent years, both in toxicology and ecotoxicology, for ecotoxicology and subsequent environmental risk assessment, the long held principles of concentration addition still seem to provide a generally reliable, albeit conservative, estimate of toxicity, with worse than additive (synergistic) effects being rare (ECETOC, 2001; Kortenkamp *et al*, 2009). This means we can usually predict the toxicity of mixtures, for risk assessment purposes, when either the chemical components of a mixture are known or it is characterised through summary parameters. These risk assessments tend to focus on defined mixtures, such as chemical products (petrochemical mixtures, pesticides, biocides, etc.) or perhaps on those chemicals considered likely to be released together or to co-occur in the environment. However, it is clearly more problematic to assess the potential interaction of chemicals in mixtures when not all the components are known and to determine the potential impact of all chemicals present in the environment. This can leave industry vulnerable to criticism, in particular, for not determining whether chemicals present in the environment, including those at concentrations below their respective predicted no effect concentrations (PNECs), act additively to cause an overall effect, the so-called "something from nothing" effect (Silva *et al*, 2002).

Despite some chemical mixtures (types 1 and 2) being regulated as products, there is a widely held belief that chemical risk assessment is done solely on a single chemical by chemical basis and that in doing so, the risk to the environment is underestimated. However, this is not necessarily the case. As stated, some chemical mixtures are regulated and risk assessed as products. Furthermore, in risk assessment, uncertainties are accounted for by the use of assessment or safety factors (e.g. in the derivation of PNECs). The Technical Guidance Document (TGD) (EC, 2003) lists one of the uncertainties as *"laboratory data to field impact extrapolation (additive, synergistic and antagonistic effects from the presence of other substances may also play a role here)"*. Lepper (2005), in discussing the setting of Environmental Quality Standards for Priority Substances under the Water Framework Directive (WFD) (EC, 2000), states "*Quality Standards derived by the proposed methodological framework do not account explicitly for a possible combined action of pollutant mixtures. Nonetheless, it is assumed that the safety factors applied in the effects assessment do cover the possible occurrence of combined action of pollutants in most instances to a great extent"*. Therefore it may be assumed that in many instances for prospective risk assessment, mixture effects are accounted for in the conservatism of the assessment process and that any further assessment factor is unnecessary. Additional conservatism in the prospective risk assessment process could result in the loss of benefits from a new chemical use, or unwarranted mitigation costs and should not be introduced without good justification and evidence that the current procedures are inadequate.

Current European monitoring programmes, e.g. the EU–Funded, EU Framework 6 NORMAN project (network of reference laboratories for the monitoring of emerging environmental pollutants, http://www.norman-network.net/index_php.php), increasingly highlight the issue of exposure of aquatic environments to mixtures of chemicals. As the sensitivity of analytical techniques increases, so does the number of chemicals detected, increasing these concerns. In addition, as the WFD is implemented, where water bodies are classified as being below good ecological status, chemicals and mixtures of chemicals can be expected to be considered guilty by association as suspected agents causing adverse effects. REACH (EC, 2006) does not address this question directly since it is driven by prospective risk assessment or simple hazard profiling, neither of which explicitly consider the combined action of unknown mixtures in the environment. NoMiracle (NOvel Methods for Integrated Risk Assessment of CumuLative stressors in Europe), another EU Framework 6 project, had amongst its objectives “to develop new methods for assessing the cumulative risks from combined exposures to several stressors including mixtures of chemical and physical / biological agents” and “to develop a research framework for the description and interpretation of cumulative exposure and effect” (http://nomiracle.jrc.ec.europa.eu/Pageslib/Objectives.aspx).

As it is impossible to consider, or even predict, the ever-changing potential combinations of chemicals in the environment, the potential for prospective risk assessment of mixtures, beyond perhaps some mixture products and other, well-defined mixtures, will be limited. Furthermore, as discussed above, it may be assumed that the potential for combined effects is covered by the assessment factors already used. However, retrospective approaches that compare predicted risks from chemicals as well as other factors, e.g. in-stream habitat and altered hydrology, to measured biological quality (e.g. structure and function) can provide an integrated assessment that gives a relative measure of the importance of chemical mixtures in causing adverse biological responses. In essence, these retrospective approaches provide an ecological reality check by identifying priority concerns pertinent for appropriate management of water quality, including Environmental Quality Standards set within the WFD. However, all this is not a simple activity and requires development of methods to discriminate impacts of chemicals (or other stressors) from natural environmental variation.

Since both the chemical industry and the water industry have stakes in ensuring good water quality, this approach may facilitate future co-operation, i.e. a wider multi-sector involvement in understanding the true impact of chemicals and the effectiveness of treatment infrastructure. To develop this retrospective approach further an ECETOC Task Force was commissioned with the following Terms of Reference:

- Review field based approaches for assessing impacts on the aquatic environment and develop guidance on suitable methods.
- Using case studies, identify research needs, including how methods can be implemented, what diagnostic tools are required.
- Consider the value of retrospective assessment in assessing environmental capacity for future industrial development.

2. EUROPEAN REGULATORY FRAMEWORK OF MIXTURES IN THE ENVIRONMENT

As mentioned in the "The European Environment and Health Action Plan 2004-2010" (EC, 2004), the assessment of the overall environmental impact on human health has to become more efficient by taking into account effects such as: cocktail effects, combined exposure, and cumulative effects. Whilst this particular statement focuses on human health, it brings focus onto concerns for environmental mixtures.

To date, this recommendation has not led to any European legislative initiatives that specifically address mixtures, other than the already existing Directive 1999/45/EC on Classification Packaging and Labelling of Dangerous Preparations (EC, 1999) and its successor CLP Regulation EC 1272/2008 (EC, 2008a). These two legislative frameworks only address the hazard communication for mixtures or preparations that are placed on the European market and as such these do not address the potential impacts of these mixtures when released into the environment.

In Annex III of Directive 1999/45/EC, a method for the evaluation of the environmental hazards of preparations is provided that is comparable to the Concentration Addition (CA) methodology that was proposed for inclusion into the REACH Regulation. However, in the published version of this regulation (EC, 2006), the annex on this topic was no longer included. Hence, although under the REACH regulation the assessment of mixtures is foreseen, there are no specific methodologies included that address how this has to be performed. In part 4.1.3 of Annex I in Regulation EC 1272/2008, the use of additivity formula is included in a tiered approach to classification of mixtures for acute and chronic (long-term) aquatic environmental hazards.

Another piece of legislation that is likely to look into the effects of mixtures in the receiving environment is the Water Framework Directive (WFD) (EC, 2000). This directive has the objective to protect inland, transitional and coastal waters throughout Europe with the aim of achieving a 'good quality status' by 2015 regarding the chemicals present and the ecology. The shift towards the ecological status can be seen as a fundamental change in the assessment of water bodies in the EU. However, to date, the guidance developed for the implementation of the directive, as well as the subsequent directive on Environmental Quality Standards (EQS) (EC, 2008b), only address single substances and apply a risk assessment methodology that is comparable with the approach adopted for new and existing substances (EC, 2006).

These EQSs are derived for a number of Priority Substances that Member States are obliged to monitor in the aquatic environment and for which the environmental concentrations must be below the defined threshold, the EQS. For several substances, that are named Priority Hazardous Substances (PHS) these EQSs are the first objective to be met with the ultimate aim of total removal of these from the environment by 2028.

As part of the Common Implementation Strategy (CIS) of the WFD a series of guidance documents has been developed, including one for the derivation of EQSs (EC, 2011). This document will be a guideline supporting the WFD implementation and will not have a legal status.

In the EQS-TGD, two methods are motioned that could serve for EQS derivation. These are the Toxic Unit (TU) approach and the Toxic Equivalent concentration approach. The first is applicable to mixtures that are qualitatively and quantitatively well defined, whereas the second is applicable to grouped substances that exert a similar mode of action.

Furthermore, the EQS guidance addresses a method that can be applied for the derivation of the hazard of substances that are of unknown or of variable composition, are complex reaction products or are biological materials (UVCBs). The specific example mentioned is petroleum products that comprise of complex mixtures of hydrocarbons. By using the PETROTOX model of Concawe (HydroQual Inc., 2008), the HC_5 (hazard concentration for 5% of species) for a product can be estimated. However, the guidance is very clear that this value cannot be used to set an EQS, as UVCBs are often comprised of constituents that have different physico-chemical properties and exhibit different environmental fate and behaviour. Hence, the composition in the environment differs from that of the substance, which is a generic property of mixtures and specifically UVCBs.

The fact that the WFD is a directive allows the Member States to go beyond the aforementioned key objectives. The Danish EPA has, therefore, released a guideline on the evaluation of waste water toxicity that includes a risk assessment of mixtures which applies concentration addition to an effluent by summing the ratios of substance concentrations and their respective PNECs (Tørsløv *et al*, 2002). Hence, the assessment of mixtures under the WFD is a possibility that may get further attention in the future. As indicated above, the estimated EQS-equivalent is a surrogate of the mixture toxicity at the point of emission, as the fate of the components and the dilution in the receiving environment will alter the environmental concentrations and the consequent PEC/PNEC ratios and, therefore, any potential for impact.

During the 2988th Environment Council meeting on 22 December 2009 in Brussels, the European Commission was invited to pay more attention to the exposures to mixtures and Chemical Cocktails and report back to the Council by early 2012 on an assessment of EU legislation and its adequacy to address risks from exposures to multiple chemicals from different sources and pathways. This was followed by a preliminary joint opinion from the Scientific Committee on Consumer Safety (SCCS), Scientific Committee on Health and Environmental Risks (SCHER) and Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) on the Toxicity and Assessment of Chemical Mixtures (EC, 2011).

3. MIXTURE TOXICITY

It is not the intention of this report to provide a comprehensive review of mixture toxicity theory as this was included in an extensive review of the aquatic toxicity of chemical mixtures undertaken and reported previously by ECETOC (2001). The use of modelling to predict the toxicity of mixtures would, however, allow implementation of prospective risk assessment schemes when environmental mixtures can be reliably predicted. This type of prospective assessment will include product assessment, where the product is already a mixture such as petrochemicals or pesticide formulations and also where joint emissions can be adequately predicted and quantified. However, it needs to be understood that once released into the environment, relative concentrations of the constituents and the associated risk, will change. In addition, it should be possible to use mixture toxicity theory, together with environmental monitoring of chemicals to indicate whether the biological condition of an aquatic environment could be explained on the basis of predicted toxicity of the chemical mixture.

3.1. Mixture Toxicity Theory

Whilst the terminology associated with the joint actions of chemicals can be variable, the basic concepts were described by Plackett and Hewlett (1952), based on Bliss (1939). The table below, taken from the ECETOC review (2001) describes the different interactions.

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 the 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

Whilst the above approach is commonly accepted, it should be noted that even Plackett and Hewlett highlighted that it has limitations. Some of the limitations are a consequence of the scheme being mathematically based rather than biological, as discussed by de Zwart and Posthuma (2005). Firstly, whilst the simplification of separating the types of joint action into distinct categories works as a mathematical concept, the biological systems being dealt with are complex, with the results observed being the consequence of several separate, overlapping processes. Similarly, the assumption that a chemical only has one target site is a simplification and finally, the classification into the different joint action classes relies on knowledge of the mechanism of toxic action, something which is rarely known or is necessarily constant as it may vary at different concentrations, target sites and indeed differ between taxonomic groups. Despite its limitations, this model is acknowledged as the basis for predicting and analysing mixture toxicity.

Initial mixture toxicity concerns were related to the potential for synergistic interactions. However, despite much research in this area and many reviews of the ecotoxicity of mixtures, the consensus is that synergistic interactions are rare (ECETOC, 2001; Kortenkamp *et al*, 2009, EC, 2011). There are some well-known examples, such as that between P450 inhibitors and insecticides, which has been researched and reported in a wide variety of terrestrial and aquatic organisms (Pilling, 1992; Johnston *et al*, 1994; Cedergreen *et al*, 2006; Adam *et al*, 2009; Bjergager *et al*, 2011). This is an example of metabolic synergism, the mechanism of which is well understood. Indeed, this synergism is used commercially with piperonyl butoxide being used as a synergist in certain insecticide formulations, which allows the reduction of active ingredient, reducing the overall environmental and resistance risk (Keane, 1998). Another example of apparent synergy is where one chemical, for example a surfactant, aids the uptake of a toxic chemical, thus increasing the internal dose. Again, this is used to increase the efficacy of pesticide and biocide formulations with the potential to reduce the overall environmental burden, but clearly the potential for effects on non-targets of the mixture, not just the active ingredient, needs to be evaluated.

Synergistic interactions are therefore rare and antagonistic interactions are of no particular concern with respect to risk. Reviews of mixture toxicity have long concluded that for risk assessment the focus should be on the non-interactive models, normally referred to as concentration addition [CA] and independent action [IA] (EIFAC, 1980; ECETOC, 2001; DEPA, 2005; Syberg *et al*, 2009; Kortenkamp *et al*, 2009).

CA is presumed to be the appropriate model for chemicals which have the same site and mode of action, whilst IA is appropriate for dissimilar acting chemicals. One important difference is that using CA, all chemicals, regardless of their concentration will contribute to the overall mixture toxicity. In contrast, IA predicts that chemicals present below the level at which they have any individual effect, will not contribute to toxicity. Thus with CA, mixing chemicals together at or below their NOECs may result in effects, the so-called ‘something from nothing’ (Silva *et al*, 2002). Following IA, mixing chemicals below their true no effect level will not result in effects. However, interpretation of data can be difficult, as although chemicals may be below a statistical NOEC, they may be above the 'true' no effect concentration and the mode of action may not be fully understood. Walter *et al* (2002) tested the effects of a mixture of 11 aquatic priority pollutants, combined at their NOECs, on algae. The inhibition of algal growth was 64%, compared to predictions of 58 and 100%, based on IA and CA, respectively and so IA was the best predictor. Of course, mixing together of chemicals at equitoxic amounts is interesting experimentally, but will not occur in the environment. In a more realistic scenario, Junghans *et al* (2006) mixed together 25 pesticides at their predicted environmental concentrations and demonstrated an effect on algal reproduction of 46%, compared to predictions by IA and CA of 39 and 49%, respectively. This study demonstrates two important points. The first is that the toxicity of the mixture was dominated by a few of the 25 pesticides. Secondly, although CA may be the better predictor as these dominant chemicals were herbicides with a similar Mode of Action to photosystem II inhibitors, the difference between IA and CA predictions for this mixture at more realistic environmental concentrations is small.

Because mixtures of chemicals in the environment will contain many chemicals which are not well characterised with respect to mode of action, CA is considered to be the appropriate conservative approach to evaluate mixture toxicity with respect to environmental risk assessment (EC, 2009). Whilst this may be considered the default assumption, it is likely to be strictly true only for chemicals which act via non-polar narcosis (baseline toxicity). Chemicals which exert a more specific mechanism of toxicity are likely to have a threshold concentration below which they do not exert any toxicity, other than contributing to baseline toxicity. However, as demonstrated above, for many environmental mixtures, the differences between IA and CA may be irrelevant given the other uncertainties within the risk assessment and CA is an appropriate initial default.

3.2. Mixture Toxicity in Regulation

Where mixture toxicity is currently applied in EU environmental regulation, a CA model is generally applied. For example, the Dangerous Preparations Directive, 1999/45/EC (recently replaced by REACH) used CA of the individual components to derive hazard classifications (R50/51/52). It is also an element of the tiered approach to classification of mixtures for acute and chronic (long term) aquatic environmental hazards (i.e. Acute Category 1 and Chronic Categories 1 to 4) under the CLP regulation EC 1272/2008 (EC, 2008a) and Globally Harmonised System (GHS) of Classification and Labelling (UN, 2009). Relevant ingredients for environmental hazard classification of mixtures are those present in a concentration equal to or greater than 0.1% (w/w) for ingredients classified Acute 1 and/or Chronic 1 and equal to or greater that 1% (w/w) for other ingredients, unless there is a presumption (e.g. in the case of highly toxic ingredients) that an ingredient present at a concentration < 0.1% can still be relevant for classifying the mixture for aquatic environmental hazards. The CLP and GHS tiered approach for classification of mixtures allows the use of 'summation' methods, bridging principles and aquatic toxicity test data available on the mixture as a whole. However, EC_{50} or LC_{50} data for the mixture can only be used to determine acute aquatic toxicity. They cannot be used to classify a mixture for chronic aquatic environmental hazards or to derive a PNEC, since dissipation and partitioning in the environment will make the comparison with PEC (Predicted Environmental Concentration) meaningless.

The Technical Guidance Document for Deriving Water Quality Standards (EC, 2011) discusses the setting of EQS values for grouped substances that exert a similar mode of action and proposes a toxic unit (TU) approach which is based on CA. Whilst it proposes the approach for substances with a similar mode of action, it will also be important to ensure that the individual EQS values are based on the same toxicity endpoint.

For plant protection products, following the guidance on risk assessment for birds and mammals, acute toxicity of mixture products is generally assessed assuming CA (EFSA, 2009), which in part, is due to animal welfare considerations to reduce animal testing. It is also worth noting that it cautions against the use of mixture toxicity approaches to evaluate long-term and reproductive risks, without very specific knowledge of the mechanism of toxic action.

One of the best established examples of concentration addition is the hydrocarbon block method (Concawe, 1996). Many petroleum substances, although described by single CAS numbers are mixtures of hydrocarbons, which will have potentially different environmental fate and ecotoxicological properties. These hydrocarbons can be blocked together based on these properties for the environmental risk assessment. These blocks can then be summed together assuming concentration addition, an assumption that is valid for these hydrocarbons as they act via baseline toxicity.

3.3. Other Approaches to Mixtures Toxicity

Mixture toxicity theory as explained above can be used for predicting the toxicity environmental mixtures. However, a major limitation of this method is that it requires knowledge of the composition of the mixture, which is not always the case. Experimental bioassay approaches can be used where the exact composition is unknown. One recommended experimental method for testing the toxicity of multi-component substances which contain poorly water-soluble components (i.e. UVCB products not 'preparations') is the use of water-accommodated fractions (WAFs), (Girling *et al*, 1994; Rufli *et al*, 1998; ECHA, 2008). WAFs allow the toxicity of a complex substance to be determined while minimising the potential for physical effects resulting from un-dissolved test substance. The observed toxicity reflects the multi-constituent dissolution behaviour of the constituents comprising the complex substance at a given substance to water loading. Application of WAFs includes the toxicity testing of petroleum oils, dispersants and dispersed oil (Singer *et al*, 2000) for use in classification and labelling.

Another bioassay method has been used to evaluate toxicity due to unknown organic toxicants in Dutch inland waters. The method has three stages:

- Concentration procedure: solid phase extraction using XAD resins, followed by elution using acetone, before distillation to remove the acetone and redissolving in water.
- Toxicity testing: selected bioassays include Microtox, algae, rotifers, *Thamnocephalus* and *Daphnia* and can include acute and chronic effects.
- Interpretation of results: determination of whether the location sampled is ‘at risk’, based on single species and/or species sensitivity distributions (SSDs).

The details of the method are given in Durand *et al* (2009). Results are expressed in terms of ‘toxic pressure’, a relative term. Although derived from laboratory data it is assumed that it is an indication of the potential for effects on natural ecosystems. Using the Microtox assay, it had been shown that the net toxicity in the rivers Rhine and Meuse had decreased during the 1990s. Bioassay monitoring using the battery of organisms described in Durand *et al* (2009) have shown that toxic pressure decreased throughout the period of 2000-2009 in the Rhine, Meuse and Scheldt (Struijs *et al*, 2010). Thus in this system at least, the potential for toxic effects from any combination of unknown chemicals has decreased over the last decade.

Whilst the idea of baseline toxicity and concentration addition is generally accepted as a unifying concept for non-polar organic chemicals, the situation with chemicals exhibiting more specific modes of action is less clear. A key tenant in understanding mixture toxicity is the relationship of external exposure to internal dose, mechanism of action and resultant toxicity. Several authors (Escher and Hermens, 2002; McCarty and MacKay, 1993) have described the relationships of critical body burdens or tissue residues to mixture toxicity. Using concepts such as these, Dyer *et al* (2000a) assessed the potential toxicity of metals (11) and organic pollutants (12) measured in 2878 fish collected from 1010 sites throughout the state of Ohio. The study included various methods of assessing mixture toxicity, including the use of critical body burden (e.g. water quality criterion*BCF = CBB), concentration addition for all toxicants and addition based on molar units for organics. For metals, three approaches were used as well, each based on different methods to base toxic units:

1. Screening values determined from the Environmental Residue Effects Database (U.S. Army Corps of Engineers: www.wes.army.mil/el/dots/dots.html) assuming a consistent hardness value (50 mg/L $CaCO_3$);
2. the 5th percentile of literature data;

3. benchmarking metal residue levels in fish occurring in Ohio locations with excellent index of biotic integrity scores (IBI >45). This last approach is only applicable for metals, which are ubiquitous and often essential.

In the end, it was quite clear for organic contaminants that CA-based approaches overestimated biological effects and that only the concept of baseline toxicity seemed to be validated. That is, only one fish in the 1010 sampled exceeded the chronic baseline toxicity threshold. Not surprisingly, there was a more closely aligned relationship of measured fish community biotic integrity with in-stream habitat variation than with the estimation of toxicity due to mixtures. Importantly, the overestimate of mixture effects within this study can only be exacerbated when one considers that these fish were exposed to a wider myriad of potential toxicants. Hence, it is strongly concluded that CA approaches indeed serve as conservative approach for estimating toxicity. Further, the tissue screening values determined for metals accomplished the same effect – that is they were conservative when addition rules were applied to each of the three different methods.

There is an increasing interest in approaches which look at mixture toxicity in terms of how chemicals are taken up, distributed, metabolised and excreted (toxicokinetics, TK) and how they interact with the target receptors (toxicodynamics, TD). Knowledge of the physiological and biochemical pathways and biological receptors is important to understand mixture toxicity. It can inform whether CA or IA are indeed appropriate and can be particularly informative with understanding those rare cases of synergism or antagonism. With the exception of vertebrates and perhaps endangered species, the focus in ecotoxicology is on population and ecosystem level endpoints rather than the individual. This, together with the difficulty of conducting TKTD studies in aquatic invertebrates because of their small size, limits the availability of data. There are some studies, for example Ashauer *et al* (2007) use TKTD approaches to investigate the effects of exposure of *Gammarus* to two insecticides, carbaryl and chlorpyrifos, evaluating a semi mechanistic model, the Threshold Damage Model for multiple toxicants (TDM_{mix}). However, data are often limited to total body-burdens as in the case of Dyer *et al*, discussed above. Nevertheless, measurement of body-burdens can have value as an indicator of environmental exposure to chemicals either singly or in mixtures.

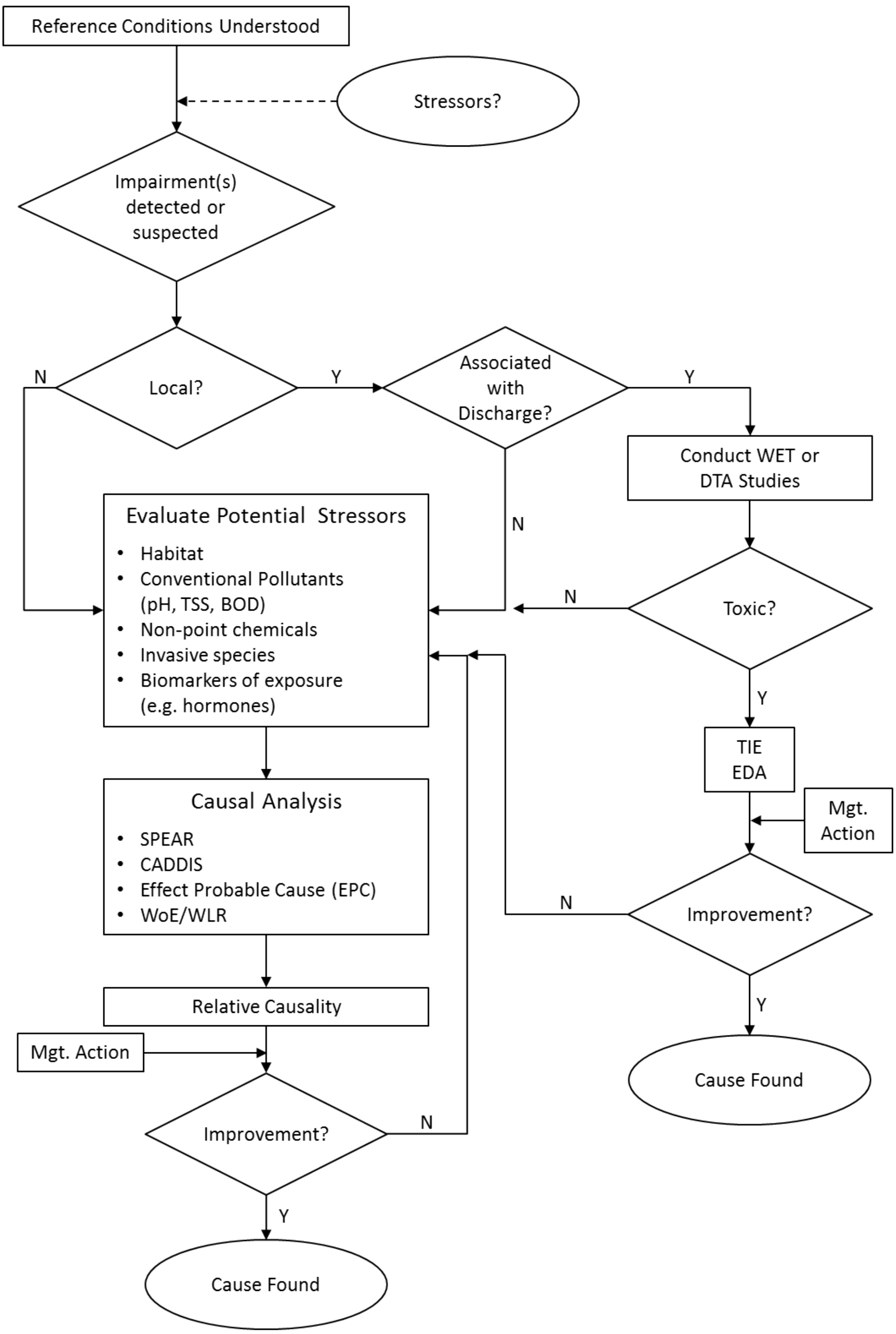
4. ASSESSING THE POTENTIAL IMPACT FROM CHEMICAL MIXTURES IN THE ENVIRONMENT

As discussed in Chapter 1, prospective risk assessment of mixtures in the environment is likely to be limited to well-defined mixtures, which can and should be managed under the respective EU directives covering different chemical sectors (PPPs, biocides, industrial chemicals). Furthermore, some point-source discharges can be well characterised, assessed and managed. However, problems may result from the unintentional mixture(s) of chemicals from diverse anthropogenic and natural sources, some of which will have the potential to adversely impact aquatic ecosystems. Assuming the status of aquatic biological communities provides a measure of impact, it is reasonable to conclude that concentrations of chemicals and their mixtures in non-impacted areas are acceptable. Contrarily, if it can be shown that the status of these aquatic communities is adversely affected, then it is reasonable to investigate the potential of the impact to be due to the presence of chemicals.

Ecological status is dependent upon understanding the relative relationships of impacted to non-impacted sites. In order to establish whether a particular water body has been impacted it is necessary to understand what an unimpacted water body should be like. This unimpacted state is often called the 'reference condition'. An example is within the WFD where the biological status can range from high (reference condition) through good, moderate, poor to bad, based on a number of biological parameters. Since the above situations require measures of ecological impacts, the following sections incorporate retrospective-based analyses in order to determine the potentially causative factors responsible for the measured impacts.

The starting point for assessing the potential ecological impact of a chemical or chemical mixture is having an understanding of the reference condition. The reference condition can range from a 'before' condition – such as in spills, to upstream of a point source, to sites that are judged to be minimally human-affected. Deviations from reference conditions provide a measure of impact. Figure 1 shows a framework of how to consider chemical or chemical mixtures as potentially contributing causes in impacted areas. This framework will be discussed in the following sections via use of case studies.

Figure 1: Suggested approach to assessment of ecologic risk of mixtures of chemicals in the aquatic environment



For example, if an impact has a potential point-source cause, then the approaches to be used should include whole effluent toxicity (WET) testing or direct toxicity assessment (DTA) to confirm, or otherwise, that chemicals are responsible. This could be followed by Toxicity Identification Evaluation (TIE), Effect Directed Analysis (EDA) or Bioassay Directed Fractionation (BDF) to identify the chemical(s) or types of chemicals responsible for the toxicity.

If an environmental impact has no obvious probable cause, then causal analysis approaches need to be employed to investigate and determine which stressors (of which chemical mixtures are one) are the most probable cause of the impact.

4.1. Assessing ecological status

As illustrated in the previous section, the first stage in retrospective risk assessments is to assess if the site in question is actually impaired. While some assessments of ecological quality have relied on chemical analysis, this section will focus on biological methods since ecological quality-based assessments should include physical (e.g. habitat loss, flow alteration) as well as chemical factors. Hence, a chemicals-only-based approach does not reflect the ecological realities of a site’s condition. Even so, where ecological impacts are found all potential stressors, including chemical mixtures, should be considered.

An important advantage of assessing ecological condition by focusing on the biology of receiving waters, is that they can integrate the effects of a range of anthropogenic stressors and, depending on the life-span and mobility of the assemblage being monitored, they can integrate effects over time and space. Furthermore, since the ecology, i.e. structure and function of the system is often the protection goal, a more direct assessment of the biological communities may be preferred, to a more indirect measurement such as chemical monitoring. Chemical monitoring may still have a significant role to play, but more in the analysis of the cause of the ecological impairment rather than the degree to which the test site is impaired.

The EU Water Framework Directive has a requirement to evaluate both the chemical and ecological status of all waters in the EU by 2012. In response to this need, there has been increased interest in methods for evaluating the ecological status of surface waters, prompting several European projects exploring the measurement of the river ecological status including the STAR, AQEM, ECOFRAME and Modelkey projects (Brack *et al*, 2005; Furse *et al*, 2006; Hering *et al*, 2004; Moss *et al*, 2003). Many of these projects highlight the need to develop an expectation of ecological condition based on minimally-impacted sites, i.e. the reference condition. The following sections describe the reference condition concept and how it is used to assess impact to aquatic biological communities.

4.1.1 Reference condition

A range of methods have been developed to assess the ecological status of a site or the degree to which a site is impaired. Most of the more recent methods share the concept of the reference condition, where the ecological community present in water bodies is minimally impaired by human activity and used as a baseline for comparison with test sites (Hawkins *et al*, 2010). The ecological community present at the test site is compared to that, which would have been expected if the site had been in reference condition. When assessing ecological status for WFD purposes, the degree to which a community at the test site differs from the reference condition is expressed in terms of an Ecological Quality Ratio (EQR) where the result observed in the test site is divided by the value expected under reference conditions. Bands or classes of conditions can be defined based on the resulting scores to indicate different degrees of impairment (Karr and Chu, 2000).

In methods comparing biological communities by species diversity and abundance, reference sites (or reference conditions) are usually selected or developed specifically for the site or stream type under investigation. This enables discrimination between sites (with different typology or environmental characteristics) that may naturally score poorly in terms of species richness and those which are impaired or degraded. In a recent review, Hawkins *et al* (2010) described the commonly used approaches for matching test sites to potential reference sites. Perhaps the most common approach divides the study area into regions of relative homogeneity with similar climate, landform, soil, geology, natural vegetation and hydrology, such as ecoregions or ecotypes (Hawkins *et al*, 2010). This typology can be refined by identifying groups of sites e.g. different types of river. The assumption is that the sites within this typology are relatively homogeneous, allowing test sites and reference sites to be compared to each other. Another approach uses site-specific predictive methods, where the community present at a test site is compared to the predicted community under a reference condition based on the relationships between environmental characteristics and biota.

Within the area of interest, candidate reference sites have been identified on the basis of expert judgement (Clarke *et al*, 2003), chemical analyses (Malloy *et al*, 2007), proximity of potential stressors (e.g. different land-use), or a combination of these. Some studies have used scoring methods based on the degree and type of local human activity, e.g. using a Geographic Information System (GIS) to identify the potentially least impacted water bodies to use as reference sites (Ode *et al*, 2005; Pont *et al*, 2007; Yates and Bailey, 2010). One of the main difficulties faced by reference condition methods is the lack of appropriate reference sites covering the full range of characteristics of test sites. For example, it may be especially difficult to find reference sites in lowland areas which tend to be more heavily populated. Further, large rivers often prove to be problematic as they tend to receive inputs from diversely affected tributaries, direct inputs from industrial and municipal discharges, have channelisation for shipping purposes, and diverse effects from urbanisation.

The most common methods for analysing biological data to determine the degree of impact fall broadly into two approaches, multi-metric indices, and RIVer invertebrate Prediction And Classification System (RIVPACS) type methods. These methods have been compared and contrasted in several reviews (e.g. Reynoldson *et al*, 1997). Many ecologists have noticed that species traits often coincide with certain physical factors in a stream, such as insects and fish may be dorsally-ventrally flattened in fast flowing streams. Relationships of traits to a myriad of ecological factors have been developed with the goal of using traits instead of traditional taxonomic methods (van den Brink *et al*, 2011).

4.1.2 RIVPACS type methods

In the RIVPACS-type methods, multivariate statistics are employed to predict the composition of the community in test sites based on the communities present in sites with similar environmental characteristics. The assumption being, that the ecological quality of a test site is determined by the degree of similarity between a test site and the relevant reference sites (Feio *et al*, 2007). The earliest of these methods was RIVPACS, developed for UK rivers (Clarke *et al*, 2003; Wright, 1995). Related approaches have since been developed for Australia, (AUSRIVAS, AUStralian RIVer Assessment Scheme) (Simpson and Norris, 2000) and Canada (BEAST, BEnthic Assessment of SedimenT) (Reynoldson *et al*, 1995), Sweden (SWEPACSRI, SWEdish Prediction And Classification system for Stream Riffle Invertebrates), the Czech Republic (PERLA, named after the stonefly genus *Perla*) (Davy-Bowker *et al*, 2006), the Mediterranean (MEDPACS, MEDiterranean Prediction And Classification System) (Poquet *et al*, 2009) and for intertidal rocky shores (O'Hara *et al*, 2010).

Candidate reference sites are selected to encompass a wide range of physical types of water bodies across a geological region and may be further refined by eliminating outliers if they correlate with evidence of human activity (Clarke *et al*, 2003; Feio *et al*, 2007). The reference sites are grouped based on their biological attributes, grouping sites with statistically similar community structure (Feio *et al*, 2007). Discriminant analysis is then used to determine which of the environmental variables best discriminates between the different assemblage groupings and to derive the equations that relate the environmental variables to the biological grouping. These environmental variables are carefully selected to include only those unlikely to be influenced by human activity. This enables the matching of test sites to reference sites purely on the basis of their natural variables. Test sites are given the predicted probability of belonging to different reference groups based on their environmental predictor variables using discriminant analysis (Clarke *et al*, 2003). The predicted (expected under reference conditions) and measured community for the test site can then be expressed in terms of an observed / expected ratio.

In these RIVPACS-type methods the degree of ecological impairment is therefore related only to changes between the assemblages measured at tests sites and those expected under reference conditions and no use is made of specific stressor gradients. This means fewer assumptions are made about the potential stressors acting on the environment. However, these methods do appear very data intensive (at least initially); requiring reference sites covering the gradient of most environmental variables likely to be encountered at test sites, i.e. a huge initial activity to develop a set of reference sites that cover all regions / possibilities.

The RIVPACS-type methods could be considered to be the first of a two-phase process, only informing us if the site is impaired and how much. The next stage would need to be the identification and assessment of potential stressors (section 4.4, Causal Analysis / Ecological Diagnostics).

4.1.3 Multi-metric indices

A multi-metric index (MMI) is a combination of metrics representing different community properties which are combined into one value to indicate the overall quality of the test site. The first of these methods was the Index of Biological Integrity (IBI) developed by Karr (1981) for fish in the USA in which scores were based on species richness and composition, trophic composition and fish abundance and condition. This approach has since been extended and developed for other environments e.g. lakes, estuaries and Mediterranean streams, and other assemblages, e.g. benthic invertebrates and macrophytes (Beck *et al*, 2010; Magalhaes *et al*, 2008; Malloy *et al*, 2007).

More recent MMIs are region specific with a number of metrics selected from a longer list of candidate metrics usually based on their ability to differentiate between different pre-defined classes of impairment or their relationship with stressor gradients (e.g. Trigal *et al*, 2009). Candidate metrics can include those related to species composition or abundance, richness or diversity, pollutant sensitivity or tolerance, or metrics based on the ecological function of taxa, with an emphasis on those metrics which respond monotonically to environmental stress (Korte *et al*, 2010).

Once the candidate metrics have been calculated, those best able to discriminate between the sites' pre-determined impairment levels (either impaired / reference or graded categories) are identified. In addition, when the index is also used to aid in the identification of potential stressors, which is often the case for MMIs, the metrics most responsive to abiotic gradients and gradients representing potential stressors are also identified. The short-listed metrics are checked for responsiveness and redundancy, selecting those responding predictably to a stressor or impairment gradients. In essence, metrics can be considered biological community-level traits. Here, reference sites may be used to determine how an individual metric changes in response to a stressor gradient and for redundancy (or co-correlation), selecting the one with the highest explanatory power (Beck *et al*, 2010; Ode *et al*, 2005). The final metrics are then combined to form the index, e.g. by transforming the metrics to unit-less values and averaging them to form the MMI. The range of MMI values may be divided into different classes of impairment by comparing them to the values produced by the reference sites. For example, in developing a benthic macroinvertebrate index of biological integrity (B-IBI), boundaries between sites of fair and poor status (the impairment threshold) were defined as two standard deviations below the mean of the index at the reference sites and the other boundaries were equally divided into 3 classes above; very good, good and fair, and 2 classes below; poor and very poor (Ode *et al*, 2005).

The final MMIs can be checked by assessing an independent data set, comparing its MMI result to its pre-classified result based on its stressor scoring. Again, this assumes the thresholds defined for pressure classes are the same as its ecological status. The FAME project acknowledges this limitation and has recommended more efforts on defining initial classification thresholds on biological criteria such as irreversibility, recovery time and extinction risk (Schmutz *et al*, 2007).

In addition to determining the overall level of ecological impairment, the main advantage of MMI methods are that they are a more integrated approach, the results from the individual indices can indicate which stressors are acting on the impaired environment and can therefore be used in the causal analysis investigations described later (section 4.4).

Multi-metric indices in which most of the component metrics have been selected for their predictive power for particular stressor types may be limited if inappropriate surrogates are used to indicate certain stressors and if not all the relevant stressors are represented. In addition, there does appear to be more reliance on methods which rely on pre-classification of sites (predefined impairment classes).

4.1.4 Trait-based methods

A relatively new, but rapidly developing field involves the use of monitoring methodologies based on traits to link ecological status with the impact of stressors. Traits describe the physical characteristics, ecological niche, and functional role of species within ecosystems (Baird *et al*, 2008) and so can provide a more direct link to environmental stressors than traditional taxonomy-based methods. For a bioassessment, specific traits are defined as a character or measurable property of an organism, e.g. sensitivity to certain stressors, the dispersal ability, the time to reproduction, the presence of the organism during the application of pesticides, growth rate, body size, etc. According to Culp *et al* (2011), the linkage of trait responses to stressor gradients promises to expand biomonitoring approaches beyond traditional taxonomic-based assessments that identify ecological effect, to provide a causal diagnosis. In common with the methods described above, the principle for the trait-based biomonitoring is the sampling of wildlife from potentially impacted and reference sites. To assess the level of impact of a stressor differences in taxonomic composition between the samples are evaluated and compared. As published by Culp *et al* (2011), *"species identity serves as a surrogate for the suite of attributes that a species possesses, with these attributes influenced by environmental conditions and evolutionary processes. An unstated assumption of this approach is that the presence of a species indicates that it possesses the traits necessary to cope with environmental conditions at a site. Alternatively, the traits of taxa present at a site can be used to indicate environmental conditions, and further, infer mechanisms by which the community composition is shaped"*. A comprehensive review of the trait-based ecological risk assessment was undertaken and reported in the SETAC Traits-based Ecological Risk Assessment (TERA) project. (van den Brink *et al*, 2011).

One example for the bioindicator method based on ecological traits is SPEAR (SPEcies At Risk) (Liess and von der Ohe, 2005). SPEAR was originally developed for detecting effects of pesticides on invertebrate communities in freshwater streams. It brings a link between the level of exposure of insecticides and fungicides and invertebrate community structure in lotic waters. Traits in the SPEAR approach are based on the sensitivity of organisms to organic toxicants, generation time, migration ability, presence of aquatic stages during maximum pesticide exposure. The SPEAR model classifies and groups monitored data of freshwater invertebrates into species that are likely to be affected by a certain stressor (species at risk) and those that are likely to be resistant (species not at risk) according to the defined biological and physiological traits (Liess *et al*, 2008). It has been shown that SPEAR is relatively independent of abiotic environmental factors other than pesticides (Liess and von der Ohe, 2005; Schriever and Liess, 2007).

The initial SPEAR indicator is based on macroinvertebrate data at species-level (Liess and von der Ohe, 2005; Schäfer *et al*, 2007; Schriever and Liess, 2007; Beketov and Liess, 2008; Liess *et al*, 2008). Biomonitoring at the level of species can be time consuming and costly. For this reason Beketov *et al* (2009) adapted the SPEAR model on the basis of a family-level. The authors reported that the predictive power of the family-level index was only slightly lower than that of the species-level index. Furthermore, the research demonstrated that the SPEAR values were not influenced by the region where the monitoring took place. Beketov and Liess (2008) also developed $SPEAR_{organic}$, a biomonitoring model that is specific for organic toxicants (e.g. synthetic surfactants and petrochemicals) with a more constant exposure regime compared to the $SPEAR_{pesticide}$ model with a pesticide specific short-term exposure regime.

As the SPEAR approach is based on species or family traits, it is not restricted to use in one geographic area, as are conventional bioassessment models using taxonomic composition or abundance parameters. SPEAR has been applied in different biogeographical regions in Europe (Liess and von der Ohe, 2005; Schäfer *et al*, 2007; von der Ohe *et al*, 2007). Furthermore, in the study by Schäfer *et al* (2010), the SPEAR model was modified with a trait-based index for salinity stress for South-East Australia. The authors investigated the power of the trait-based approach to identify and disentangle effects of salinity and pesticides. Selected traits were the generation time, reproduction mode, dispersal capacity and physiological sensitivity. According to the authors the trait-based indices allow the differentiation between multiple stressors.

Trait-based approaches have significant potential over the more traditional taxonomic methods. Baird *et al* (2011) explain that as stream communities from disparate regions should be functionally similar they should therefore have predictable and consistent sets of traits associated with particular habitat types. Consequently trait-based comparisons may be able to overcome some of the problems experienced using the taxonomic based approaches (e.g. RIVPACS-type and MMI), such as difficulties in the identification of appropriate reference sites (of sufficiently high ecological status in the region of interest) and the extrapolation across wider areas due to biogeographic differences in community species composition.

4.2. Whole Effluent Toxicity (WET) testing and Direct Toxicity Assessment (DTA)

4.2.1 Whole Effluent Toxicity testing

Whole Effluent Toxicity (WET) testing is a well-established method of assessing the potential impact of discharges, which potentially contain mixtures of chemicals, on the aquatic environment. The USA introduced the Clean Water Act, 1972 with the objective of *"restoring the chemical, physical and biological integrity of the Nation's waters"*. WET testing was one of the tools developed as part of a programme of further legislation to regulate and control discharges in aquatic environments. WET testing is a bioassay technique to determine the acute and chronic toxicity of a whole effluent, potentially containing a number of chemicals which may contribute to overall toxicity, to a battery of test organisms. These test organisms have been selected on the basis of their suitability for laboratory testing and on their environmental relevance and cover both freshwater and saltwater organisms (US EPA, 2002a,b). The test organisms are exposed to a range of concentrations of an effluent, from 100% downwards, and EC/LC_{50} and NOECs expressed as the % effluent concentration. A previous ECETOC report contained a review of WET testing and its implementation into Whole Effluent Assessment (WEA) schemes (ECETOC, 2004).

A recent example of WET testing being used to test for mixture effects comes from Sowers *et al* (2009).

Based on the hypothesis of the potential oestrogenic effects of municipal effluents on fish, a two-generation study with fathead minnow exposed to municipal effluent at 0%, 50% and 100% effluent was conducted. Survival, growth, reproduction and histological aspects were studied.

The results showed that this wastewater effluent did not pose a significant threat to the survival, growth, and reproduction of the fathead minnow parent generation, despite a reduction in secondary sexual characteristics of 100% wastewater-exposed males. Males in the F_1 generation exhibited an accelerated sexual development, although the underlying mechanism for this observation remains unknown.

4.2.2 Comparing WET to in-stream biological effects

Whole effluent testing is a tool to determine potential toxicity of an effluent, but there still needs to be a link established between the toxicity of the effluent and environmental impact. A comprehensive study comparing WET to in-stream biological effects was conducted by Diamond and Daley (2000). The authors compiled a database of 250 dischargers across the United States and examined relationships between standardised *Ceriodaphnia dubia* and *Pimephales promelas* (fathead minnows), WET test endpoints, and in-stream biological condition as measured by benthic macroinvertebrate assessments. Sites were included in the analysis if the effluents were not manipulated before testing (e.g. dechlorination), and standardised biological and physical habitat assessment methods were used upstream and directly downstream of the discharge. Several analyses indicated that fish endpoints were more related to in-stream biological condition than *Ceriodaphnia* WET endpoints. Dischargers which showed toxicity in approximately a quarter of their tests had a less than 15% chance of exhibiting in-stream impairment. Effluent dilution was the strongest factor affecting relationships between WET and observed biological conditions. Effluents that comprised greater than 80% of the stream under low-flow conditions exhibited stronger relationships between whole effluent toxicity test failures and in-stream condition than effluents with greater dilution. Effluents that comprised less than 20% of the stream had a low probability of exhibiting impairment, even if several WET test failures were observed over a one-year period. Fish acute and chronic WET information could predict in-stream biological conditions; however, WET compliance, based on 7Q10 (critical low flow – 7 lowest flow days in a 10-year period) stream flow, was consistently conservative. The authors' results indicate that WET was more predictive of in-stream biological condition if several tests were conducted, more than one type of test was conducted, and endpoints within a test were relatively consistent over time.

A comparison of acute whole effluent toxicity (daphnid and algae) from municipal, agromunicipal and agroindustrial WWTP effluents to receiving water IBI (Index of Biotic Integrity) scores was conducted by Ra *et al* (2006). WWTPs utilising activated sludge treatment recorded fewer toxicity violations compared to rotating biological contactors and extended aeration basins, respectively. WWTPs with consistent toxicity failures resulted in the greatest impairments to fish biotic integrity. In particular, WWTPs receiving manure from agricultural sources more often experienced WET failure, probably due to increased ammonia concentrations.

Diamond *et al* (2008) conducted a preliminary, yet highly thorough, study to determine the relationships between chronic whole effluent toxicity test results from 6 municipal WWTPs using the fathead minnow, *Ceriodaphnia dubia* and *Pseudokirchneriella subcapitata*, with in-stream periphyton, invertebrate and fish communities data. The types of treatment used per WWTP were not specified, yet given the hydraulic retention periods of three of the plants it appears reasonable that they used activated sludge in their treatment train. The other three had long hydraulic retention times (>87 d) or were not reported. Hence, the extrapolability of the study based on treatment types would be dubious. Nevertheless, the study was conducted from data over a 1.5-year period from six different sites across the USA. A data-quality-objectives approach was used that included several proposed measurement quality objectives (MQOs) that specified desired precision, bias, and sensitivity of methods used. The six facilities all had design effluent concentrations >60% of the stream flow (at 7Q10, or stream design flow). In addition to at least quarterly chronic *Ceriodaphnia dubia*, *Pimephales promelas,* and *Selenastrum capricornutum* (green algae) WET tests, other tests were conducted to address MQOs, including splits, duplicates, and blind positive and negative controls. Macroinvertebrate, fish, and periphyton bioassessments were conducted at multiple locations upstream and downstream of each facility. The test acceptance criteria of the US Environmental Protection Agency (US EPA) were met for most WET tests; however, this study demonstrated the need to incorporate other MQOs (minimum and maximum per cent significant difference and performance on blind samples) to ensure accurate interpretation of effluent toxicity.

More false positives, higher toxicity, and more ‘failed’ (noncompliant) tests were observed using NOECs than when using the IC_{25} endpoint (concentration causing 25% decrease in organism response compared to controls). Algae tests often indicated the most effluent toxicity in this study; however, this test was most susceptible to false positives and high interlaboratory variability. Overall, WET test results exhibited few relationships with bioassessment results even when accounting for actual effluent dilution. In general, neither frequency of WET noncompliance nor magnitude of toxicity in tests were significantly related to differences in biological condition upstream and downstream of a discharge. Periphyton assessments were the best at discriminating small changes downstream of the effluent, followed by macroinvertebrates and fish. Although sampling methods were robust, more replicate samples collected upstream and downstream of each facility were needed to increase detection power.

4.2.3 Upstream – Downstream of WWTP Studies

Dyer and Wang (2002) reported on a study designed to identify broad relationships that may have relevance for the risk assessment of chemicals and materials that are discharged to receiving streams via municipal wastewater treatment plant (WWTP) effluents (e.g. consumer product ingredients). The effects of municipal wastewaters occurring in high population density (>500 persons per square mile, urban) and low population density (<500 persons per square mile, rural) environments were determined via analysis of biological, habitat, and water chemistry data collected both immediately upstream and downstream of 221 WWTPs in Ohio, USA. Further, per cent cumulative effluent at both low (7Q10) and annual mean flows were calculated for all sites, acting as a surrogate for all potentially unmeasured contaminants present in effluent and discharged to receiving waters. Several biological and chemical indicators demonstrated poorer water quality in urban areas compared to rural areas. After considering the effect of river size, adverse effects downstream of WWTPs for both fish and macroinvertebrate communities were clearly identified for only urban areas. These data indicate that WWTP potency may be greater in urban areas compared to rural areas.

Several suggestions were proposed to explain the increased adverse effects observed at urban sites compared to rural sites. First, streams in urban areas were already stressed, and the addition of wastewater and contaminants led to significant adverse responses. Supporting this assessment was the fact that upstream urban sites had mean toxic units from metals and ammonia (utilising concentration as a conservative estimate of risk) exceeding unity and had a greater per cent cumulative effluent than rural sites. While this linkage of calculated parameters to aquatic communities is not equivalent to assessing causality, it does represent a potential toxicological explanation for the adverse responses. Second, urban municipal wastewater is more potent than rural municipal wastewater inputs. Significantly increased values of cadmium, copper, lead, phosphorus, and toxic units were observed downstream in urban settings, whereas these were not found to be significant in rural areas.

In contrast to the adverse effects of urban WWTPs, adverse effects did not appear to be delivered via rural WWTPs. Certainly, municipal effluent represents complex mixtures of nutrients and contaminants, including residual concentrations of consumer product chemicals. Given the large numbers of unmeasured factors in effluent and that the mean cumulative per cent effluent at low flow for 63 of the 71 rural sites exceeded 63%, one might have expected to observe adverse biological responses, albeit less than urban areas. However, this was not observed. Their analysis confirmed Diamond and Daley’s (2000) observation that being consistently toxic and comprising greater than 80% of the river flow is more important than the per cent effluent.

4.2.4 Direct toxicity assessment

Direct toxicity assessment (DTA) was developed in the UK during the early 1990s from the whole effluent toxicity (WET) approach well-established in the USA and Canada. A toxicity test is conducted on a whole sample (such as an effluent, receiving water, or bottom sediment) to obtain a measure of the total toxicity. Identification of the many individual substances in the sample is not necessary and any additive toxicity resulting from combinations of substances with similar modes of actions is also measured. The whole toxicity measurement can be directly related to observed biological effects in the receiving environment and can be used to identify the sources of toxicity avoiding the expense of chemical identification.

A collaborative demonstration programme, involving environmental regulators and water and manufacturing industries, was carried out during 1997-2000 to trial a methodology for using DTA for the assessment and control of complex effluents in the UK. The methodology was underpinned by a seven-step protocol and a suite of short-term exposure bioassays. The sequence of steps in this protocol is shown below:

- Step 1: selection of sites for investigation.
- Step 2: toxicity screening of effluents.
- Step 3: ranking of effluents for further evaluation.
- Step 4: toxicity characterisation of effluents + site-specific risk assessment.
- Step 5: validation of predicted risk.
- Step 6: toxicity reduction.
- Step 7: monitoring.

The trial was conducted at three project sites where there was evidence of receiving water impact, and ecotoxicity from complex effluents was a probable contributing factor:

- A reach of the River Aire near Bradford in Yorkshire – previous work had revealed poor receiving water quality with the effluent from the Esholt sewage treatment works (STW) having been shown to be toxic to invertebrates in short-term exposure bioassays.
- A reach of the River Esk near Langholm in the borders between Scotland and England – historical data indicated a reduction in the diversity of invertebrates downstream to the Langholm STW and the effluent from the works was toxic to aquatic invertebrates in short-term exposure assessments.
- The lower Tees estuary on the north-east coast of England – both historical and recent evidence of poor environmental quality (including evidence of receiving water ecotoxicity) and ecotoxic effluents entering the estuary.

The outcomes of each project are summarised in a paper by Tinsley *et al* (2004). Detailed final reports for each project are also available (cited by Tinsley *et al*, 2004), along with learning points and recommendations on how to best use bioassays for the assessment and control of complex effluents in the UK (UK WIR, 2001a), and guidance on how to carry out the bioassays and on how to use the data generated for regulatory decision-making (UK WIR, 2001b).

Chemical analysis, biological survey work and bioassays were carried out for each project. The results for the River Aire showed neither the receiving water nor any of the effluents caused short-term exposure toxicity to invertebrates (*Daphnia magna*) and that the quality of the

Esholt STW final effluent had improved as a result of improvements made to the treatment process in recent years. Thus no further work was carried out at the site. However, concurrent to this work, longer-term exposure bioassays using the feeding rate of invertebrates (*Gammarus pulex*) as a sublethal endpoint were deployed in the river, which indicated a restricted invertebrate community both upstream and downstream of Esholt STW. It was concluded that this was a result of poor water quality (including sublethal ecotoxic effects caused by longer-term exposures to mixtures of chemicals) and bioavailable sediment contamination.

The work on the Esk project, showed little evidence of reduced invertebrate diversity or short-term exposure toxicity to invertebrates in the river downstream of the discharge, although the Langholm sewage treatment works final effluent was found to be consistently toxic to aquatic invertebrates (*D. magna*), except during a short period in the summer when traders were on holiday (i.e. indicating source of toxicity was industrial waste). However, invertebrates deployed in the river (*G. pulex*) showed reduced feeding rates downstream of the discharge. It was concluded that the treated sewage effluent was diluted and dispersed in the river with the possibility of only sublethal effects on invertebrates and more marked effects under low flow conditions. Short-term exposure bioassays were then used to track back to the source of ecotoxicity within the sewer network. Two potential candidate sources were identified (organophosphate pesticides and surfactants) (Hutchings *et al*, 2004). Options for reducing or removing the ecotoxicity problem, including a waste minimisation approach, were explored and evaluated.

The Tees is an industrial catchment receiving high volumes of complex waste from a number of industrial and domestic sources. The larger industrial and sewage treatment works effluents were screened using short-term exposure invertebrate (oyster) bioassays and all but one were found to be toxic, despite compliance with their chemical specific discharge licence limits. A dilution and dispersion model was used to assess the extent and distribution of ecotoxicity in the surface waters of the estuary. A field survey revealed short-term exposure toxicity in the estuary, which was in line with model predictions. A study designed to demonstrate that effluent toxicity measured at the ‘end of pipe’ can be tracked back to identify the source and nature of the toxicity was undertaken on a combined domestic sewage and industrial effluent entering the estuary via an open channel. High throughput versions of the short-term exposure algae and invertebrate bioassays were used successfully for this purpose. The toxicity tracking showed that considerable toxicity in the final effluents was associated with untreated industrial discharge. This has subsequently been diverted to treatment. Toxicity identification evaluation (section 4.3) highlighted cyanide as responsible for the measured toxicity (Hutchings *et al*, 2004).

WET testing and DTA are undoubtedly useful, cost effective tools which will give information on the inherent toxicity of effluents or environmental samples, independent of any specific chemical analysis. However, it is not a direct assessment of environmental impact, there is still the question of extrapolation to the environment, both with respect to the dilution in receiving watercourses for effluents and extrapolation from the toxicity endpoints in the WET testing / DTA to biological effects in the environment. For example, acute toxicity endpoints may not be sufficient to identify effects caused from longer-term exposures. Furthermore, they are only an indication of toxicity, rather than identifying the specific cause. Following the scheme in Figure 1, further work may be necessary to identify cause, before any management procedures are implemented.

4.3. Toxicity Identification Evaluation (TIE) and Effects Directed Analysis (EDA)

Toxicity Identification Evaluation (TIE) or Effects Directed Analysis (EDA) is the logical next steps following WET/DTA. To successfully manage and mitigate the environmental impact following the demonstration of toxicity from effluents or environmental samples it is necessary to identify the toxicant or toxicants responsible, isolate the source, evaluate the effectiveness of control options, and confirm toxicity reduction.

Both TIE and EDA use biological tests as indicators of toxicity and, through manipulation of the sample, characterise the chemical or chemicals responsible for toxicity. The aim is to reduce the complexity of the mixture through physical and chemical manipulations of the sample and, through biotesting, identify which fraction is associated with the toxicity. Further analysis of the fraction(s) is done until finally chemical analysis can identify those compounds potentially responsible for toxicity. A confirmation step, again with further testing and weight of evidence approaches can confirm the identity of the toxicants, would seem to be necessary before any management steps are implemented to control the suspected toxicants.

The US EPA TIE scheme is described in a number of EPA publications (US EPA 1991, 1992, 1993a,b, 2007) and relies on whole organism toxicity testing. EDA approaches include whole organism testing, but often rely on *in vitro* testing methods (Bakker *et al*, 2007; Hecker and Hollert, 2009). Both TIE and EDA methods have been applied to investigate toxicity in water and sediments. More than 30 case studies describing various aspects of the toxicity reduction evaluation (TRE) process with specific attention directed to TIE procedures are included in the proceedings of the 2001 SETAC workshop on “Toxicity Identification Evaluation: What Works, What Doesn’t...” (Norberg-King *et al*, 2005).

The first application of the TIE approach to study toxicity in aquatic sediments is reported to be the study of Ankley *et al* (1990). Sediments were collected from the lower Fox River / Green Bay in Wisconsin and measured for various biological and chemical parameters. Sediments from the system contained an extremely complex mixture of persistent inorganic and organic contaminants including metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls and pesticides. Phase I, II and III TIE analysis of the pore water from the Fox River / Green bay sediments revealed a relatively unexpected result with respect to the causes of toxicity in the samples. Despite the presence of a number of anthropogenic contaminants in the sediments, the chemical responsible for the pore water toxicity was ammonia, which is generated in sediments by microbial process involved in the degradation of nitrogenous organic materials. The identification of an eutrophication product as opposed to persistent contaminants as a toxic compound would result in very different risk management decisions.

The discussion paper of Ankley and Mount (1996) uses the above study with contaminated sediments to illustrate the basic principles and assumptions of the two main approaches for dealing with multiple chemical exposures: model-based or empirical methods. In addition to the empirical TIE approach described above, they describe model-based approaches as using toxicological relationships to predict biological effects based on contaminant concentrations measured in the environment. They cite the work of Swartz *et al* (1995), who established a 'ΣPAH' model that predicts the combined toxicity (survival in 10-day exposures) of PAHs in sediments to the marine amphipod, *Rhepoxynius abronius*.

A combination of toxicity tracking and TIE was used in two of the case studies in the UK DTA Demonstration Programme (River Esk and Lower Tees Estuary) to enhance the understanding of the source and type of toxicants present. The results are summarised in the paper of Hutchings *et al* (2004). The River Esk case study is described here since it provides an example where predicted ecotoxicity (based on additivity of the identified substances) was compared with measured ecotoxicity. Toxicity was assessed using the acute *D. magna* immobilisation test, which had previously been identified as the most sensitive bioassay for this case. TIEs were completed on 23/9/98 and 9/3/99 on the Langholm STW effluent. Reduction in ecotoxicity was achieved by pH adjustment and C18 solid phase extraction on both occasions. Methanol fractions eluted from the C18 columns were confirmed to be toxic to *D. magna*. These fractions were analysed by gas chromatography linked to mass spectrometry (GC/MS) and found to contain significant quantities of the organophosphate pesticide diazinon. The measured concentration of diazinon was sufficient to fully explain the measured base-line ecotoxicity on the first sampling occasion (23/9/98) but not sufficient to explain baseline ecotoxicity on the second sampling occasion (9/3/99). During the second TIE, aeration of the STW effluent at its initial pH also resulted in toxicity reduction. Consequently it appeared that substances other than diazinon were responsible for the overall STW effluent ecotoxicity and that the toxicity of these substances was reduced by aeration. Since removal of ecotoxicity is consistent with the presence of detergents, which can become attached to the glassware during the aeration process, it was postulated that although pesticides alone could not explain whole effluent ecotoxicity the presence of surfactants may have enhanced toxic effects in a synergistic manner. Toxicity tracking was conducted throughout the sewer system from 12 sampling points. Both treated and untreated samples were tested. Although a reduction in the levels of pesticide could be seen chemically, no reduction was observed in ecotoxicity suggesting that other components were contributing to the toxicity. It was concluded that further toxicity tracking would be required to establish the true picture of toxicity sources within this system.

The value of TIE as a diagnostic tool is the idea that the toxicity of a complex environmental sample can be identified to a specific fraction of the whole. This is an indication that rather than a complex issue, involving chemical cocktails and many interactions, the toxicity is often associated with a few, or even a single chemical being responsible for the majority of the toxicity. Thus environmental management is likely to be successful focusing on a small number of chemicals, rather on a wide range of chemicals which contribute little to overall toxicity.

4.4. Causal analysis / Ecological diagnostics

Techniques such as WET, DTA, TIE and EDA are useful to determine the cause of any adverse environmental effects, but they rely on there being a likely probable cause identified, for example an effluent or other discharge. However things are not always so clear cut and it may be that there is an observation of an undesirable biological effect (e.g. fish kill, decline in biological index, high incidences of abnormalities, etc.) with no obvious cause. Nevertheless, the cause of the biological impairment must be determined in order to design appropriate remedial management actions. Chemicals, either singly or in mixture, are often cited as the probable cause of adverse environmental quality, but to draw this conclusion it is necessary to establish a causal link between the chemicals and the measures of ecological status. The null hypothesis is that the additivity of chemical mixtures will be proportional to adverse ecological impacts. However, biological communities do not simply respond only to chemical stressors, but are responsive to changes in other factors that affect water quality such as nutrients, dissolved oxygen, suspended solids, and conductivity, among a myriad of other factors. These factors can be the result of a number of environmental perturbations such as land-use (e.g. urbanisation, agriculture), hydrological modification (e.g. dams, dikes, channelisation) and in-stream habitat (presence of diverse substrates, riffle / runs / pools, shade). The investigation of causality of chemical mixtures must take into account the potential effects of these other factors.

The study of relating chemical-induced effects relative to other factors has been referred to as causal analysis, ecological diagnostics or eco-epidemiology. Whatever the terminology, the primary goal is the same, to ascertain the most important factors related to measured ecological impacts. While this is a retrospectively-based assessment, it requires tools that forecast the potential effects of both chemical and physical factors. These forecasting tools are based upon retrospective assessments where respective factors are statistically understood. For instance, the basis for identifying that a site is impacted is that the biological community deviates from the expected condition. Since habitat dictates the numbers and types of niches that may be inhabited by aquatic organisms, deviations must take habitat quality into account. Determining expectations on species presence and/or abundance requires the definition of a reference condition. Hence, all ecological diagnostics are basically investigations where the potential causes of deviation from reference condition are determined.

The aim of the following sections is to describe the diverse methods and studies that have recently been developed for the purpose of ascertaining the primary causes of ecological impacts with special consideration of chemical mixtures as a causative suite of agents. Mixtures are represented as toxic units (assumes concentration additivity), the ms-PAF (multi-substance potentially affected fraction) of species – which employs both concentration addition and response addition principles for aquatic communities, as well as the simple occurrence of effluents and their coincidence in space to measured ecological impacts.

The theories and methodologies for causal analyses as applied to environmental and health matters have recently been reviewed in a series of articles (Wickwire and Menzie, 2010 and references therein). The need for formal procedures to minimise the uncertainties associated with understanding cause and effect relationships has led to the development of organised decision support frameworks. The Causal Analysis / Diagnosis Decision Information System (CADDIS, http://www.epa.gov/caddis/) is an on-line system for identifying potential causes of aquatic biological impairments. It is based on the US EPA's Stressor Identification (SI) process, which draws from multiple types of eco-epidemiological evidence. An essential part of the CADDIS development strategy has been the use of case studies. At the time of writing, twelve examples are listed on the CADDIS website with references to manuscripts and links to US EPA reports, which are available to download. The five most recent final US EPA reports were released in June 2010. The case studies provide examples of how some assessors have developed and interpreted evidence to determine causes of biological impairments, and in some cases improved the quality of an ecosystem. Most of the cases assess rivers and streams, but a few assess terrestrial ecosystems.

The CADDIS process consists of the following steps:

- Step 1: Define the case.
- Step 2: List candidate causes.
- Step 3: Evaluate data from the case.
- Step 4: Evaluate data from elsewhere.
- Step 5: Identify probable causes.

During steps 3 and 4, candidate causes may be deferred to simplify the causal analysis and focus the assessment on more likely causes. Deferred candidate causes are revisited if a likely cause is not identified. The *Sources, Stressors, and Responses Volume* of CADDIS provides some suggestions on what to look for when deciding whether to include a candidate cause on your list. (http://www.epa.gov/caddis/ssr_home.html). Background is provided on 14 commonly encountered sources, stressors and responses: metals, sediments, nutrients, flow alteration, temperature, ionic strength, dissolved oxygen, pH, physical habitat, urbanisation, ammonia, herbicides, insecticides, and unspecified toxic chemicals.

CADDIS defines toxic chemicals as individual chemicals or mixtures of chemicals and their by-products that originate from human activities. Unspecified toxic chemicals may be unknown because they have not been measured or measurement is difficult (e.g. due to episodic occurrence, unique chemistry, or low concentrations). Their effects may be suspected but, because of no or incomplete chemical monitoring data, exposure cannot be confirmed.

Case studies which have included toxic chemicals as a candidate cause include:

- Little Floyd River, Iowa (Haake *et al*, 2010; US EPA, 2010a) – no fish were observed during a 1990 stream-use assessment, and only pollution-tolerant benthic macroinvertebrate were found;
- Touchet River, Washington (Wiseman *et al*, 2010; US EPA, 2010b) – altered benthic invertebrate assemblages and low numbers / absence of salmonids;
- Willimantic River, Connecticut (US EPA, 2010c) – altered benthic invertebrate assemblage.

In the first two case studies, known toxicants (such as pesticides, pesticide residues and polychlorinated biphenyls) were deferred because they were not detected or detected at concentrations lower than those established for chronic aquatic life. In the Little Floyd River study, ‘other toxicants’ were deferred as a candidate cause because of lack of data. It was also noted that the samples had not been tested for insecticides. These deferred candidate causes were not revisited because other stressors were identified as likely causes.

The primary probable causes of biological impairment in the Little Floyd River were increased deposited sediment and lower concentrations of dissolved oxygen. A total maximum daily load (TMDL) for sediment and dissolved oxygen was submitted to, and approved by, the US EPA in 2005. Fish have since re-colonised the stream.

The two dominant stressors in the Touchet River were identified as warmer water temperature and sedimentation. Low dissolved oxygen (DO) and high pH were considered probable contributing causes. Since completion of the Touchet River causal assessment, the Washington State Department of Ecology (WSDE) has continued to monitor the Walla Walla River Basin including the Touchet and has developed TMDLs, which were approved by the EPA in 2007, for temperature, DO and pH. Notably, the list does not include excess sediment. TMDLs were also introduced for chlorinated pesticides and PCBs. Although these legacy pollutants were deferred in the CADDIS assessment because they were at levels that were not likely to be toxic, they could not be refuted because episodic exposures may have been undetected.

An episodic release was proposed in the Willimantic case study, because the assessed candidate causes seemed unlikely to have produced the severe impairment observed at the site. This led to additional biological sampling, which localised the upper bounds of the impairment near a raceway previously obscured behind vegetation. A grey discharge was observed and traced to a broken sewer line from a nearby mill, which operated batch productions. Although the discharge was not chemically analysed, the effluent was associated with a National Pollution Discharge Elimination System (NPDES) permit that allowed discharge of metals, organic matter, acid, and ammonia to the publicly owned treatment works (POTW). Three years after rerouting the illicit discharge, the impaired sites reached acceptable biological conditions.

Yeom *et al* (2007) employed US EPA’s Stressor Identification Evaluation (SIE) to construct a conceptual model that related the potential constituents in municipal and industrial effluents to measured impacts to fish populations downstream of their discharges. Reference for the evaluation was upstream of the discharge points. Strength of evidence analyses were used to evaluate various habitat, chemical, biochemical and population measures in order to determine the most plausible factors responsible for biological impacts. Excess ammonia was assessed to be the most plausible factor for decreased fish population levels. On site exposure studies confirmed ammonia’s toxicity. All other potentially plausible factors were ruled out.

Chapman (2007) provides a simple guidance for determining when the discharge of effluent adversely impacts receiving water sediments and biota. The guidance utilises several lines of evidence and weights each to derive a weight of evidence for relating discharges to downstream adverse impacts. As with US EPA’s SIE and CADDIS, a reference is needed to determine the magnitude of the impact and to determine the plausibility of any line of evidence.

In summary, causal analysis or ecological diagnostics require an understanding of what species to expect in a certain geography and ecological setting (e.g. small versus large streams, slow versus fast flow, etc.) in order to establish the degree of impact at test sites. Diagnostics may utilise simple, logical tools to provide a weight of evidence where chemical mixtures may or may not be implicated. While less sophisticated than eco-epidemiological studies (section 4.5), they provide pragmatic information that can lead to logical decision-making. To date, these studies have not implicated complex mixtures as a primary factor in measured biological impacts. That said, it is clear that factors such as wastewater effluents and diverse non-point sources have been implicated as potential factors for impacts.

4.5. *Eco-epidemiological studies*

Outside the US EPA's SIE or CADDIS approaches, there have been relatively few examples of comprehensive eco-epidemiological studies, which establish likely causes of impact through an extensive analysis of the major stressors, including the potential for effects from chemicals. Published examples are discussed below to show the extent of data collection and analysis which is required to establish probable cause in the absence of direct evidence. Furthermore these studies give a good indication of the need to put the impact of chemicals into the context of other factors that contribute to measured biological impacts.

4.5.1 Ohio eco-epidemiological studies

Ohio EPA possesses the longest history of using the biomonitoring of fish and invertebrate communities to make decisions regarding water quality management, certainly within the USA and perhaps the world over. Given the frequency of sampling and geographic spread of data collection, it has created a unique dataset to explore the relationship of predicted and measured chemical concentrations and risks with biomonitored data. In essence, the Ohio data provide an opportunity to determine whether current risk assessment approaches are sufficiently conservative so as to protect in-stream biology, even as a consequence of exposure to chemical mixtures.

Since the mid-1990s, the Procter and Gamble Company (P&G) have been collaborating with Ohio EPA, specifically to obtain data and then share potential statistical relationships of biota and chemistry. A dataset covering fish, invertebrate and fish habitat was obtained from Ohio EPA and then arranged spatially via a geographic information system (GIS). Measured water quality data, such as pH, nutrients, ammonia, dissolved oxygen and metals concentrations were also obtained.

Initial investigations with fish community data and diverse environmental factors (in-stream habitat, ammonia, metals, total toxic units) focused on the Little Miami (Dyer *et al*, 1998b) and Great Miami Rivers, located in SW Ohio as well as preliminary work at the state-level (Dyer *et al*, 2000b). Since P&G's primary impetus was to determine the potential for consumer product chemicals to cause adverse effects to in-stream biota, per cent cumulative effluent at mean and low flows were calculated for river reaches throughout the state of Ohio. Cumulative effluent serves as a surrogate for down the drain chemicals, as mixtures. The source of generating cumulative effluent was the GIS-ROUT model (Dyer and Caprara, 1997; Wang *et al*, 2000; Wang *et al*, 2005). The model estimates influent, effluent and in-stream concentrations as a result of the per capita use per day of chemicals and accounting for removal via wastewater treatment and first-order in-stream loss. The original model was created at the national scale, modelling ~10,000 WWTPs and nearly 500,000 river miles, but was also fit into a spreadsheet format to enable Ohio-only modelling. Currently the GIS-ROUT model has been licensed to the American Cleaning Institute (formerly the Soap & Detergent Association) and is publically available as: iSTREEM (in-STREAm Exposure Model; http://gis2.uc.edu/iSTREEM/login.aspx).

A particularly important issue for early eco-epidemiological studies investigating relationships of biota to diverse habitat and chemical factors was the establishment of methods in which to aggregate data appropriately in geographic space. A GIS was used for data management. Some data were collected as points on a river (chemical sampling, fish and invertebrates samples) while other data corresponded to river reaches (e.g. GIS-ROUT predicted concentrations). Further, chemistry samples were often taken near easy access points (i.e. bridges), whereas biological samples were located throughout a river reach, or several reaches. To be able to work with the data, rules needed to be developed to be able to aggregate the data appropriately in space. The rules for spatial aggregation were based on the GIS-ROUT model. That is, a 'site' corresponded to data within a modelled river reach. River reaches in the state averaged approximately two miles in length based on confluences of WWTP effluent and tributaries and drinking water withdrawal sites. Initially, all data within a reach was averaged to determine relationships of biota versus chemistry and habitat (Dyer *et al*, 1998b; Dyer *et al*, 2000b). Since habitats and chemical concentrations could vary considerably with a reach, an imputation method was subsequently developed where the nearest chemistry and habitat data were imputed to nearest biological sampling location and data, within a river reach (Dyer and Wang, 2002). This greatly increased the numbers of sites in which to investigate biological versus other factors as well as provided a refinement from the previous studies. Studies (see below) using the imputation method include: de Zwart *et al*, 2006; Kapo and Burton, 2006; Kapo *et al*, 2008. Ongoing studies in Ohio continue to utilise the imputation method (http://www.waterborne-env.com/pubs_ecodiagnostics.asp).

The early eco-epidemiological studies utilised measured metals and ammonia concentrations as potential indicators of mixture effects. Toxic units were derived for all measured components based on US EPA water quality criteria that utilised ambient water hardness and pH and then concentration addition to derive the sum of toxic units per 'site'. Per cent cumulative effluent served as the surrogate for unmeasured contaminants emanating from WWTPs. Toxic units and cumulative effluent were generally found to be independent, hence could be used in stepwise regressions to determine the relative potency of each to measured fish and invertebrate community status (see Dyer and Caprara, 1997 and Dyer *et al*, 2000b for methods).

Initial results from the Little Miami and Great Miami Rivers as well as state-wide (Ohio) indicated that in-stream habitat appeared to be the predominant positive factor affecting in-stream biota. However, consistent negative regression coefficients were associated with cumulative effluent and total toxic units. Visual inspection of where negative biological effects were most commonly occurring illustrated that urban environments were particularly at risk.

This observation led to a variety of investigations regarding the importance of land-use / land-cover on biological status (Dyer and Wang, 2002; de Zwart *et al*, 2006; Kapo and Burton, 2006; Kapo *et al*, 2008a) and which are reviewed in some detail later in the section. Also, since it appeared that effluents were consistently associated with a negative effect, improvements were made to derive mixture toxicity of chemicals emanating from WWTPs, such as surfactants, antimicrobials and boron.

Modelled chemicals included surfactants such as linear alkyl benzenesulfonate (LAS), alkyl sulphates (AS), alcohol ethoxy sulfates (AES), alcohol ethoxylates (AE), the antimicrobial triclosan (TCS), and boron. Table 2 (from de Zwart *et al*, 2006) provides key parameters required to estimate exposure of these chemicals in effluent and receiving waters via GIS-ROUT. Concentrations were converted to estimates of risk to aquatic communities by comparing the predicted ROUT concentrations and measured bioavailable ammonia and metals concentrations to species sensitivity distributions for each chemical. The potentially affected fraction of species to chemical mixtures was determined assuming response-addition, also known as the multi-substance potentially affected fraction (ms-PAF) of species approach (see Traas *et al*, 2002). Figures 2 and 3 provide a two-step framework in which the ms-PAF is derived for chemicals across mechanisms of action. The ms-PAF continues to be the primary method for estimating mixture effects to aquatic communities.

Given the importance of the Ohio studies, the following section outlines, in chronological order, the eco-epidemiological studies that have been carried out in Ohio and illustrates how data development management and analyses have contributed to the current state of the science.

Table 2: Annual US consumption volumes for triclosan, linear alkylbenzenesulfonate (LAS), alcohol ethoxylates (AE), alcohol ethoxy sulfates (AES) and boron as well as wastewater treatment plant (WWTP) removals and first-order river loss rates as used for GIS-ROUT model estimations for riverine concentrations in Ohio

Parentheses below chemical names (LAS, AE and AES) refer to the average alkyl (C) and ethoxylate (E) chain lengths, respectively.

	Chemicals				
	Triclosan	LAS (C12)	AE (C13-E3.1)	AES (C13.45-E1.5S)	Boron
National volume use (metric tons)	600[1)]	303,458[3)]	141,976[3)]	268,077[3)]	4,536[3)]
Per capita use per day (g)	0.0062	3.137	1.467	2.771	0.0467
WWTP Process	**WWTP Removal (%)**				
Activated Sludge	95[1)]	99[4)]	99[4)]	98[4)]	0[4)]
Oxidation Ditch	95[1)]	99[4)]	99[4)]	98[4)]	0[4)]
Rotating Biological Contactor	95[1)]	98[4)]	99[4)]	98[4)]	0[4)]
Lagoon	95[1)]	98[4)]	99[4)]	98[4)]	0[4)]
Trickling Filter	80[1)]	80[4)]	96[4)]	93[4)]	0[4)]
Primary	30[1)]	27[4)]	18.9[4)]	0[4)]	0[4)]
In-stream degradation	**First-order river loss (d-1)**				
River Loss	0.264[2)]	0.7[2)]	31.2[2)]	24[2)]	0[5)]

1) McAvoy *et al*, 2002
2) Federle and Schwab, 2003
3) SRI, 2002
4) McAvoy *et al*, 1998
5) Dyer and Caprara, 1997

Figure 2: Step one of predicting mixture toxicity to aquatic communities: chemicals with similar modes of action

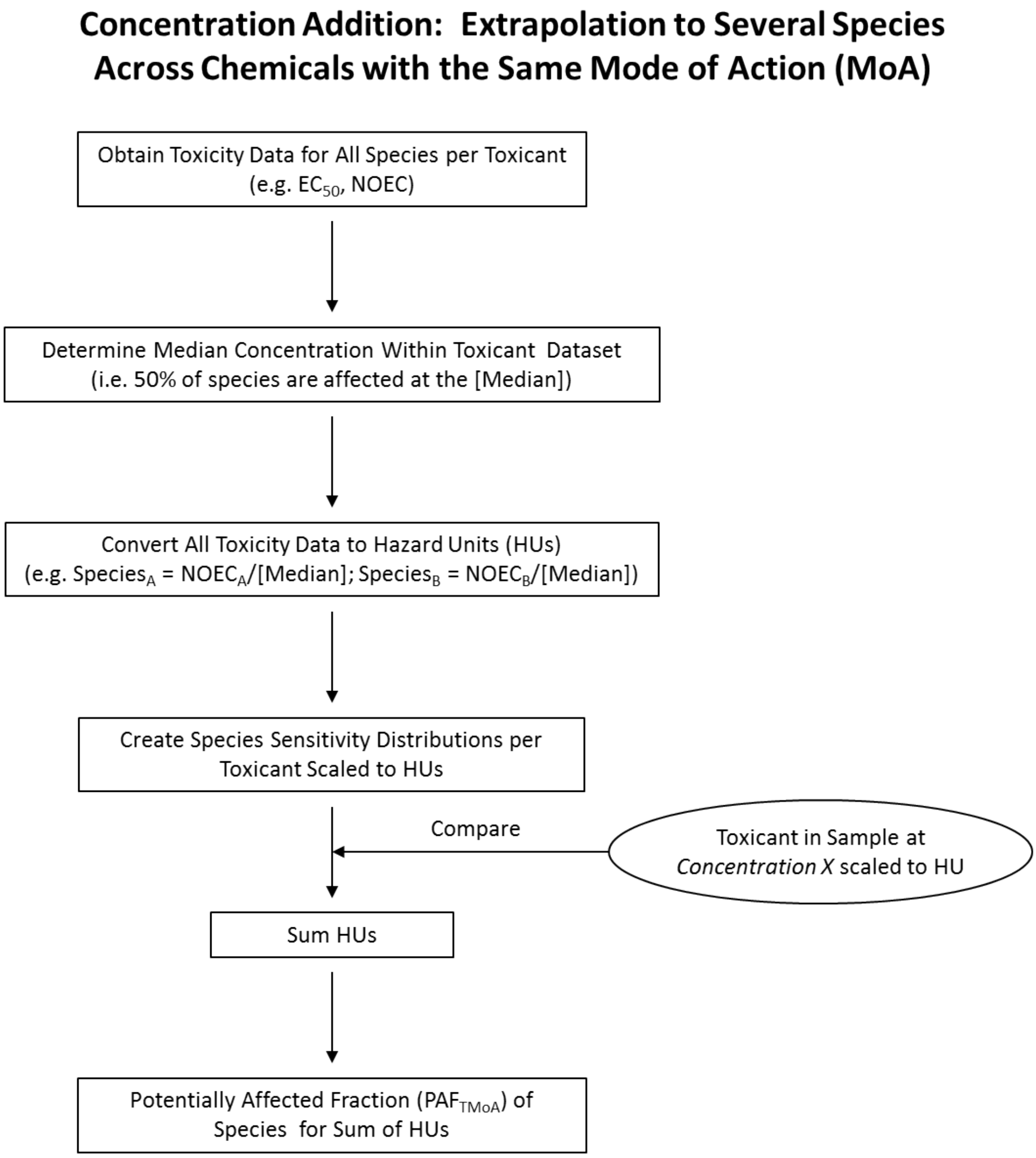
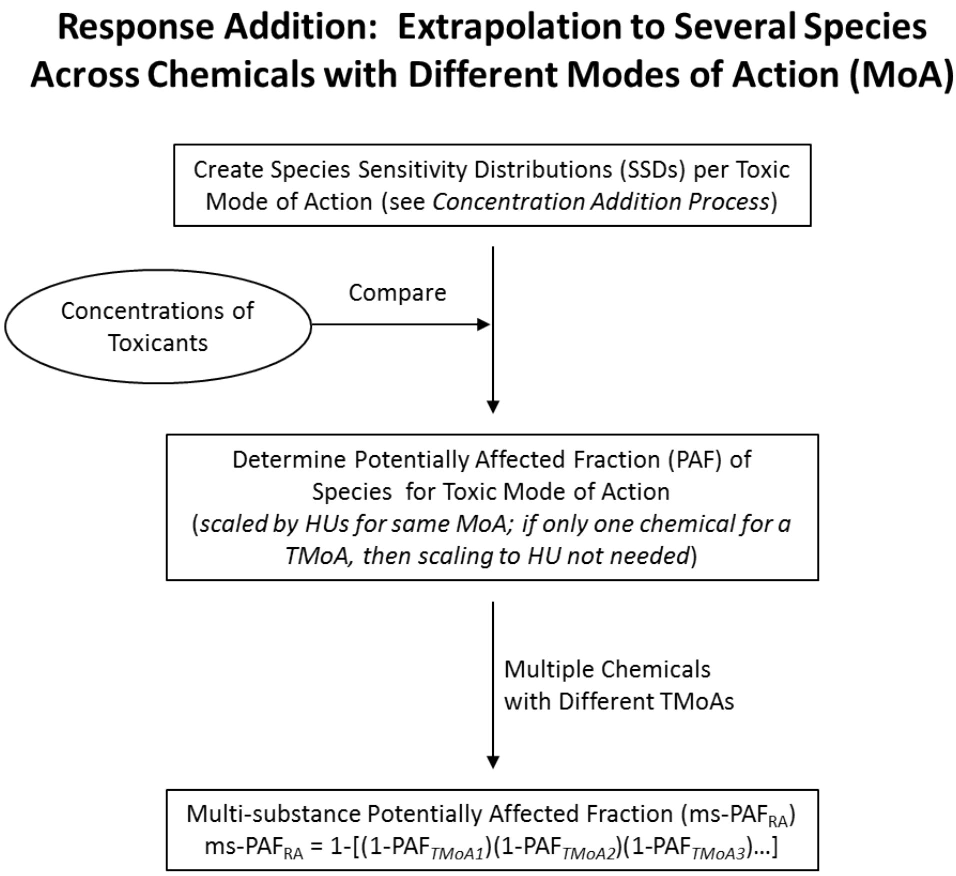


Figure 3: Step two of predicting mixture toxicity to aquatic communities: chemicals with dissimilar modes of action



The initial analysis of relating biological metrics to habitat, water chemistry and exposure to effluent was conducted by Dyer *et al* (1998a) using data collected from the Little Miami River (LMR), located in South West Ohio. The river is a National and State Scenic river, the majority of which is considered of Exceptional Warm Water Habitat by Ohio EPA. Of the discharge volume entering the LMR, 99.2% is represented by municipal waste water treatment plants (WWTPs), with only 0.8% coming from industrial sources. Mixture toxicity was assessed for measured metals and ammonia assuming concentration-addition (toxic units) and using criteria that take into account water hardness and pH. Relationships of various fish and invertebrate metrics to habitat, chemistry and effluent were developed using forward stepwise multiple linear regressions. The importance of independent factors to the biological dependent variables were determined by the forward selection step and overall model coefficient of determination. The principal environmental factors that influenced biological responses were in-stream habitat and per cent cumulative WWTP effluent at mean flow. Cumulative WWTP effluent at low flow dilution, nutrients and total toxic units from metals and ammonia did not correlate with biological responses (e.g. number of mayfly species, invertebrate community index (ICI), number of fish species and index of biotic integrity (IBI)). This study served as the pilot for a subsequent basin and state-wide analysis.

Dyer *et al* (2000) examined the relationships of fish biological community status with multiple factors, such as in-stream habitat, drainage area, gradient, cumulative effluent, conventional pollutants, and chemical mixtures, to fish communities was explored at three levels of geographical space: 1) sub-basin; 2) basin; 3) state level.

The first two approaches (termed ‘bottom-up’) focused on sub-basin and basin-level relationships within the Great Miami River, Ohio. The second approach (termed ‘top-down’) focused on state-wide relationships. Data were provided by the Ohio Environmental Protection Agency and the US EPA. These data were integrated via a GIS. Multiple linear regression was used to determine the strength of stressor–response relationships. The greatest amount of variation of the index of biotic integrity (IBI) and selected metrics was addressed at the sub-basin level, followed by the basin and state level, respectively. That is, models were most accurate at the sub-basin and basin levels and least at the state-wide level, likely due to geographical and land-use issues that needed to be accounted for models to provide significant coefficients of determination. Overall, in-stream habitat factors, stream gradient, and drainage area were the best predictors and positively related to the IBI and number of fish species. Chemical factors, such as cumulative effluent, metals, ammonia, and biochemical oxygen demand, were consistently observed as negative, moderating factors for IBI and fish taxa richness and were the best predictors of the percentage of fish observed with deformities, fin erosions, lesions, and tumours. A visual inspection of impaired sites indicated that perhaps urbanised areas may be at greater risk than their rural counterparts. A direct test of this observation was made by Dyer and Wang (2002).

The ultimate purpose of the study by Dyer and Wang (2002) was to identify broad relationships that may have relevance for the risk assessment of chemicals and materials that are discharged to receiving waters via municipal wastewater treatment plant (WWTP) effluents, such as ingredients from consumer products. The effects of municipal wastewaters occurring in high population density (>500 persons/sq. mile, urban) and low population density (<500 persons/sq. mile, rural) environments were determined via analysis of biological, habitat, and water chemistry data collected both immediately upstream and downstream of 221 WWTPs in Ohio. To create a testable dataset, data within the Ohio GIS (used by Dyer *et al*, 1998a; 2000) were manipulated such that the nearest habitat and chemistry data to a biological sampling site were imputed to the biological site so long as the habitat and chemistry data did not violate GIS-ROUT river reaches. That is, the nearest neighbouring habitat, chemistry and biological data were re-coded as a single site. More details on this are found in de Zwart *et al* (2006).

Due to the binary design of the data, student's t-tests were used to determine differences between upstream and downstream for urban and rural areas, respectively, and to compare upstream sites from urban to rural and downstream sites for both urban and rural.

Adverse biological effects from wastewater in urban areas could be generically inferred. In contrast, significant adverse effects on biological communities (fish and macroinvertebrate) downstream of rural WWTPs could not be determined. Significant differences between urban and rural sites for contaminants and total toxic units suggest that wastewater in urban areas is more potent than rural areas and/or correlates with other sources – such as storm water discharges and urbanised non-point sources. The lack of significant adverse biological effects for both invertebrates and fish downstream from rural WWTPs casts doubt on mixture toxicity issues arising from the use of down the drain products (e.g. consumer products, pharmaceuticals).

As indicated in the above studies, the method by which data are aggregated has an impact on determining the relative potency of independent habitat and chemical variables on biological metrics. In order to minimise the potential spatial variance due to aggregation within a river reach, a nearest neighbour approach, or imputation, was developed by Dyer and Wang (2002) and used in this study to investigate the potential impacts of wastewater discharges on fish and macroinvertebrate communities occurring immediately downstream and upstream from discharge points. Kapo and Burton (2006) used this dataset for the Great and Little Miami Rivers to assess a novel spatially-explicit diagnostic system: Bayesian weights of evidence (WoE) and weighted logistic regression (WLR). The WoE/WLR technique is popularly used in minerals exploration. In this study, the method was used to determine independent factors most spatially related to known impacted sites. Unlike the previous studies where a 'unit' refers to aggregated data by river reach or sites within river reaches, this method utilised a watershed approach by which land-use attributes with water flow characteristics were the prime factors for data aggregation. Imputed data (from Dyer and Wang, 2002) were used within the defined watersheds to determine the spatial proximity of various stressors to sites with known impairments. A typical watershed unit was 0.5 km^2 throughout the study area.

In-stream habitat stressors showed the greatest spatial association with biological impairment in small, low-order, streams (on average, 56% of total spatial association), whereas water chemistry, particularly that of wastewater effluent, was associated most strongly with biological impairment in high-order streams (on average, 79% of total spatial association, 28% of which was attributed to effluent). The strength of various stressors was found to also vary by land-use (forest, agriculture, urban). The WoE/WLR method provides a highly useful screening level watershed risk assessment that integrates various existing data sources and produces a clear visual communication (maps) of areas indicative of potential biological impairment and a quantitative ranking of candidate stressors and associated uncertainty.

Biological assessments should both estimate the condition of a biological resource (magnitude of alteration) and provide environmental managers with a diagnosis of the potential causes of impairment. Although methods of quantifying biological condition are well developed, identifying and proportionately attributing impairment to probable causes remain problematic. Furthermore, analyses of both condition and cause have often been difficult to communicate. De Zwart *et al* (2006) developed an approach that:

1. Links fish, habitat, and chemistry data collected from hundreds of sites in Ohio (USA) streams (imputed data from Dyer and Wang (2002);
2. assesses the biological condition at each site using a method other than multi-metric methods (e.g. index of biotic integrity);
3. attributes impairment to multiple probable causes;
4. provides the results of the analyses in simple-to-interpret pie charts.

As with previous studies, the data were managed via a GIS. Biological condition was assessed using a RIVPACS-like predictive model. The model provided probabilities of capture for 117 fish species based on the geographic location of sites (latitude, longitude) and local habitat descriptors (drainage area and river reach slope). Impaired biological condition was defined as the proportion of those native species predicted to occur at a site that were observed. The potential toxic effects of exposure to mixtures of contaminants were estimated using species sensitivity distributions and mixture toxicity principles (i.e. response-addition of species sensitivity distributions, ms-PAF, as indicated in Figures 2 and 3). Generalised linear regression models described species abundance as a function of habitat characteristics. Statistically linking biological condition, habitat characteristics including mixture risks, and species abundance allowed for the evaluation of loss of fish species with diverse environmental factors. Results for 695 sites in the state of Ohio were mapped as simple effect and probable-cause pie charts (EPC pie diagrams), with pie sizes corresponding to magnitude of local impairment, and slice sizes to the relative probable contributions of different stressors. A state-wide analysis showed that the average loss of native fish species in Ohio to be 40%. A general linear model showed that of the variance in fish species lost, the coefficient of determination was 50%. Human affected (‘dirty’) predictors associated with lost species were: water chemistry (28%); in-stream habitat quality (16%); cumulative per cent effluent (3%); and ms-PAF (toxicity, 3%). These findings were consistent with that of previous studies; however the authors believed that improvements in gathering better chemical data and land-use attributes might improve the GLM modelling. That is, the 50% uncertainty was deemed ‘grey’ and future studies should be initiated to reduce this fraction.

The leading candidate statistical analysis methods for attributing potential causality have been regression-based methods such as the EPC (de Zwart *et al*, 2006) and the spatially-explicit WoE/WLR method by Kapo and Burton (2006). Kapo *et al* (2008a) quantitatively compared both models using the same dataset from Ohio (based on Dyer and Wang, 2002 plus land-use classifications from Kapo and Burton, 2006). Direct comparisons were made for 177 impacted sites scattered throughout the state. The methods generally yielded significantly similar results in the identification of stressors and their relative influence. Greatest agreements were for (in order): urban runoff, per cent cumulative effluent, ammonia / metals ms-PAF, and in-stream habitat quality. However, key differences were also observed between the methods which reflected the distinctive objectives and sensitivities of each. The findings suggest the potential value of utilising multiple methods as independent, yet quantitative, lines of evidence in screening-level regional diagnostic assessment. An important caveat to this cross-comparison analysis is that the 177 sites that were used for comparison were those that had significant biological impairments. The vast majority of sites within Ohio did not have significant impairments (i.e. loss of fish species); hence future cross-comparisons will require a new calibration to more subtle effects, such as minor loss of species and/or loss of abundance of species.

It is clear from the above studies that eco-epidemiology requires an interdisciplinary and even inter-agency collaboration in order to determine the factors involved in biological perturbations and then translate those into appropriate mechanisms for management. While the focus of this document is chemical mixtures, such a focus without considering other factors that contribute to biological status is clearly naïve and will result in inappropriate management schemes. Posthuma and Dyer (2010) summarised recent ongoing work in Ohio where increased attention to obtaining new: (1) chemical exposure estimates (~300 chemicals: pharmaceuticals, pesticides, consumer product chemicals); (2) land-use / land-cover data; (3) biological trait data; and (4) inclusion of food-web modelling – all of which may reduce previous model uncertainties. New Ohio biological, habitat and chemical monitoring data coupled with watershed-based imputation methods allowed for an increase in the number sites to ~2000, translating into increased opportunities to explore diverse past methods as well as new. The new data set (2000-2008) illustrates a large improvement in biological status throughout the state of Ohio. More than a third of the sites are considered to be exceptional, or at reference condition. Considering there are nearly 800 WWTPs within the state, it would be conceivable that a high proportion of the river waters would be negatively affected by effluent, yet the recent analysis does not indicate such. That is, modelled mixture toxicity from the ~300 chemicals should have increased the correlation of chemical risks with biological impairments. That said it is still clear that predicted mixture effects do correlate strongly to measured biological impairments at certain sites, but this is not a widespread phenomenon. Investigations using biological traits and food webs might provide increased diagnostic capabilities beyond species reductions or compositions as the primary measures for detecting biological response to diverse environmental factors. Importantly, the early investigations clearly indicate that complex chemical mixtures do not equivalently translate into adverse impacts; hence a 'one size fits all' approach to chemical management would not be prudent. Instead, the authors suggest that the ecological contexts of chemical exposure be considered –

or plainly stated – given an ecological reality check. Chemical mixtures do occur and can cause harm to the receiving environment, but their mechanism of release, exposure and relationship to biological responses are all situation-specific. The Ohio database has served a valuable purpose to investigate not only the potential of mixtures to cause adverse biological status, but also to consider management schemes. Such data gathered over a large geography are rare. However, in order to provide realism into mixture toxicity policy, efforts to stitch together such data sets from diverse sources will be imperative for successful management.

4.5.2 Use of eco-epidemiology to determine the likely causes of poor biological quality in rivers in England and Wales

In the eco-epidemiology project by Whelan *et al* (2011) a GIS-based weight of evidence / weighted logistic regression (WoE/WLR) approach was used to assess the most likely predictors of ecological condition in 307 sites in England and Wales. This study further developed an approach used in an earlier pilot study by Kapo *et al* (2008b) published by the UK Environment Agency.

The ecological status of each site was determined using the RIVPACS methodology using macroinvertebrate data collected by the Environment Agency in the spring and autumn of 1995 and 2004. In addition to the basic site characteristics (e.g. location, altitude, slope), other potential stressors were also assessed: upstream land-use, pesticide exposure and surrogate measures for effluent exposure.

Land-use was considered at two scales: the overall fraction of the catchment occupied by different land-uses and the land-use within 100m of monitoring sites. Pesticide exposure was predicted using the SWAT model within CatchIS (Catchment Information System). In the earlier study annual average levels of pesticide exposure were used, which were not found to be predictive of ecological status. In this study, pesticide exposure was determined using the peak concentrations entering streams following the first rainfall event to initiate run-off. Concentrations were calculated using data on: soil characteristics, climate, pesticide properties, land-use and pesticide usage (the usage data was collated in 2006 and 2004). These peak concentrations were used to calculate the ms-PAF for each catchment. De Zwart *et al* (2009) describe the ms-PAF method, consistent with Figures 2 and 3. The authors describe this value as representative of the probable loss of taxa attributable to the mixture of pesticides.

The influence of sewage effluent was represented by the number of sewage treatment plants per catchment, the total population served and the loading rate of effluent into the river. The effluent loading rate was calculated based on the location, size and character of waste-water treatment plants in the catchment.

For the stressor analysis the results of the RIVPACS analysis were simplified by only using the data from those sites determined to be of high ecological status (top 25% of sites, Observed species / Expected species (O/E) ratios of >1) and low ecological status (bottom 25% of sites, O/E ratios of <0.75).

GIS-based weight of evidence analysis was used to examine the data for significant spatial associations between the biological response data (high or low ecological status) and the individual environmental variables. The threshold value at which the environmental variable was significantly spatially associated with high quality or low quality sites was also determined. These potential stressor variables were then reclassified into binary classes above and below the threshold at which the change in response was significant and the WoE analysis repeated. Of the initial nineteen individual variables – ammonia, BOD and % urban land-use had the highest association with the poor quality sites (i.e. most predictive of low quality sites).

To reduce the effects of co-correlating variables, related variables were combined into principal component association (PCA) factors. These factors were: agriculture (percentage agricultural land-use, pesticide toxicity and nutrients), urban (percentage urban land-use, ammonia and BOD), effluent (effluent loading rate, number of STPs, population served), metals and low pH, and TSS (total suspended solids).

A weighted logistic regression (WLR) model was developed to rank the predictor PCA factors according to their influence in predicting high and poor quality sites and then to predict the site condition (high or low ecological quality) from the multiple variables, mapping the probability of site status based on the spatial patterns in stressor variables.

When the model was tested against the data used to develop the model, the urban PCA (percentage urban land-use, ammonia and BOD) was the strongest predictor of condition and successfully predicted the condition of 76% of poor sites in the spring and when combined with the metals and pH PCA successfully predicted 82% of the poor sites in autumn. Of the high quality sites, the urban and metals/pH PCAs predicted 94% of high quality sites in the spring and 96% in the autumn.

Interestingly, low pesticide exposure (the low cumulative multi-substance toxicity) was predictive of high ecological quality sites but sites with high levels of exposure to pesticides were not predictive of low quality sites. The authors remark that pesticide exposure may decrease the probability of high quality sites but does not directly predict poor quality. In addition, the effluent surrogates did not demonstrate a stress-response relationship, despite the strong influence of percentage urban land-use, ammonia and BOD. This may be due to an effect of the resolution of the effluent surrogate measures. It may be interesting to consider variables such as distance from nearest upstream STP.

4.5.3 Toxic Pressure and Relationship Species Impacts in the Netherlands and Belgium

De Zwart and Posthuma (2005) utilised a mixed response addition and concentration addition suite of models (multi-substance PAF) for 261 pesticides occurring in the Netherlands to estimate the toxic pressure to aquatic communities occurring in canals and ditches. The analysis was country-wide and required information on pesticide use and runoff models for exposure evaluation. General linear models (stepwise) were used to determine the relative effects of conventional water chemistry factors (e.g. pH, Cl, total phosphorus) and pesticide mixtures (multi-substance PAF) to measured aquatic community composition at 212 sites. At the nationwide scale, only seven pesticides accounted for 96% of the predicted total species at risk. Potentially due to a low number of sites with measured biological data, the statistical models relating mixture toxicity to impacts was inconclusive, although some significant relationships were determined for some species. For example, some 'opportunists' were found to increase in areas were the impacts were predicted.

De Zwart *et al* (2009) utilised the ms-PAF approach to determine the toxic pressure to aquatic species within the Scheldt River, Belgium as a part of the EU ModelKey project (www.modelkey.org). As in the above study, GLM models were used to determine the relative relationships of chemical mixtures to other measured water quality factors to measured biological impairments. While a highly significant relationship was found with the GLM models and biological impacts, it was very clear that a considerable level of uncertainty remained, probably due to unmeasured physical and chemical factors. Therefore, the authors conclude that this analysis be considered a lower-tier result and that more work is needed for a full diagnosis.

The diagnostic deficits found in these two studies are probably due to biomonitoring data availability, understanding of reference conditions and number of sites available to populate the GLM modelling schemes rather than the methodology.

4.5.4 Use of eco-epidemiological studies to generate testable hypotheses

Diamond and Serveiss (2001) investigated the loss of mollusc (richness) and fish (IBI) in the Clinch and Powell River basins, in the Appalachian Mountains, USA. Reference sites were defined as minimally impacted by humans and were all found within the same ecoregion or physiographic province. Stepwise regressions were used to sort out which land-use characteristics were associated with the greatest impacts in both river basins. Further, analyses on sediment habitat quality (embedded-ness, riparian cover, in-stream cover, etc.) also were sorted using stepwise regression approaches. The authors note that while this statistical methodology can narrow the scope for potential management actions and create testable hypotheses, it can also identify uncertainties – such as the potential of unmeasured and analysed factors, such as water chemistry.

4.5.5 Eco-epidemiology summary

Eco-epidemiology, 'eco-epi', provides for a realistic understanding of the interplay between physical and chemical factors and their potential effects on biological status. Both retrospective and prospective fate, toxicity and risk assessment methods are employed. Further, eco-epi is quantitative, based on state-of-the-art statistical methodologies. Consequently, these studies provide a relative strength of evidence of association of diverse stressors with measured impacts that can lead to cross-cutting management methods that will incorporate both physical and chemical requirements of aquatic communities. Importantly, these assessments can be conducted at a variety of geographical scales. While complex chemical mixtures have been explicitly investigated for their role in biological impairments, in general it has been shown in state-of-the-art assessments that complex chemical mixtures do not fully explain biological impairments, however a combination of habitat and chemical factors can be used together to explain species loss in both diversity and/or abundance. Consequently, a 'one-size fits all' method for all mixture assessment and management is not warranted. Simply stated, if complex chemicals behaved the same in certain situations (e.g. domestic wastewater discharges), then the use of an assessment factor to address unknown constituents could be warranted. However, such studies have not shown this to be the case.

5. CONCLUSIONS AND RECOMMENDATIONS

Conclusions

There are methods and tools available to allow the potential risk to the environment from well characterised mixtures to be evaluated using the established mixture theory, particularly if the mode of action / mechanism of toxicity is understood. It is more problematic to understand the potential contribution to environmental impact from chemicals and/or combinations of chemicals where not all the components are known and how to evaluate this against the contributions from other stressors and environmental variables. The cause of environmental impacts on aquatic environments is a prerequisite to any remedial action. The success of procedures such as TIE/EDA and the results of other studies indicate that chemicals responsible can be identified and effectively managed. Indeed results of long-term monitoring studies, such as those from the rivers in the Dutch Delta, indicate that toxic pressure has been reducing i.e. that management measures, presumably to reduce emissions are being effective. There is no evidence to support an overhaul of current risk assessment schemes or any additional conservatism in the assessment factors in prospective risk assessment schemes.

Retrospective causal analysis and in particular eco-epidemiological studies allow the contribution of chemical mixtures to be determined. These studies can show that mixture impacts can be spatially quantified in aquatic ecosystems, and there is a need to understand site-specific stressor combinations in order to define effective measures to improve ecological status. Overall, the limited datasets available show that, whilst chemicals may be responsible for some environmental impacts, they are by no means the only or even the most important factor.

Recommendations

Two recommendations are suggested: (1) improve biological traits associated with non-perturbed sites and (2) improve diagnostics that distinguish chemical factors from physical / chemical factors responsible for biological impairments.

Regarding the first one, there is still a relative paucity of data regarding the life histories of most taxa (plant, invertebrate and vertebrate) associated with exceptional water quality. For example, the relative frequency of intersex of most fish species in the relative absence of chemical exposure is not known at present. Further, the relationships of intersex and population structure for most species are not known. In essence, basic ecological research is needed to understand reference-condition structure and function. Such research will provide the basis for refined predictions (both pro- and retrospective) for biological expectations per site and hence, a more accurate measure of biological impairment.

While many statistical and the best professional judgment approaches have been utilised to distinguish chemical effects versus other stressors with regard to diagnosing biological impairments – there still exists relatively few examples and well utilised approaches that could eventually become standard guidelines for stressor diagnostics. Issues regarding site, river reaches, catchments and regional scale assessments can require highly different methodologies, and therefore highly different diagnostics. Sufficient experience exists to begin the road toward guideline development. With appropriate diagnostic guidelines, appropriate interpretations of the importance of chemical mixtures compared to other factors can be made, therefore leading to better water quality management decisions.

ABBREVIATIONS

AE	Alcohol ethoxylates
AES	Alcohol ethoxy sulfates
AS	Alkyl sulphates
BCF	Bioconcentration factor
BDF	Bioassay directed fractionation
BIA	Biological impact assessment
B-IBI	Benthic index of biological integrity
BOD	Biological oxygen demand
CA	Concentration addition
CADDIS	Causal Analysis / Diagnosis Decision Information System
CAS	Chemical Abstracts Service
CBB	Critical body burden
CIS	Common implementation strategy
CLP	Classification, labelling and packaging of substances and mixtures
DO	Dissolved oxygen
DTA	Direct toxicity assessment
EC_{50}	Effective concentration, 50%, median effective concentration
EDA	Effect directed analysis
EPA	Environmental Protection Agency
EPC	Effect and probable cause
EPT	Ephemeroptera, plecoptera, and trichoptera
EQR	Ecological quality ratio
EQS	Environmental quality standards
EU	European Union
FAME	Future of the Atlantic Marine Environment
GCMS	Gas chromatography mass spectrometry
GHS	Globally harmonised system
GIS	Geographic information system
GLM	General linear model
HC_5	Hazard concentration, 5%
HU	Hazard unit
IA	Independent action
IBI	Index of biological integrity / Index of biotic integrity
IC_{25}	Inhibiting concentration, 25%
ICI	Invertebrate community index

LAS	Linear alkyl benzenesulfonate
LC_{50}	Lethal concentration, 50%, median lethal concentration
LMR	Little Miami River
MMI	Multi-metric indices
MoA	Mode of action
MQO	Measurement quality objectives
ms-PAF	Multi-substance potentially affected fraction
NOEC	No observed effect concentration
NPDES	National pollution discharge elimination system
PAH	Polycyclic aromatic hydrocarbon
PCA	Principal Component Association
PCB	Polychlorinated biphenyl
PEC	Predicted environmental concentration
PHS	Priority hazardous substances
PNEC	Predicted no effect concentration
POTW	Publicly owned treatment works
PPP	Plant protection products
REACH	Registration, evaluation, authorisation and restriction of chemicals
RIVPACS	RIver invertebrate Prediction And Classification System
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SI	Stressor identification
SIE	Stressor identification evaluation
SPEAR	Species at risk
SSD	Species sensitivity distribution
STW	Sewage treatment works
SWAT	Soil and water assessment tool (river basin model)
TCS	Triclosan
TD	Toxicodynamics
TDM_{mix}	Threshold Damage Model for multiple toxicants
TERA	Traits-based ecological risk assessment
TGD	Technical guidance document
TIE	Toxicity identification evaluation
TK	Toxicokinetics
TMDL	Total maximum daily load
TMoA	Toxic mode of action

TRE	Toxicity reduction evaluation
TSS	Total suspended solids
TU	Toxic unit
UVCB substance	Substance of unknown or variable composition, complex reaction products or biological materials
WAF	Water accommodated fraction
WEA	Whole effluent assessment
WET	Whole effluent toxicity testing
WFD	Water Framework Directive
WLR	Weighted logistic regression
WoE	Weight of evidence
WSDE	Washington State Department of Ecology
WWTP	Waste water treatment plant

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