Review

Effluent Complexity and Ecotoxicology: Regulating the Variable within Varied Systems

Paul A. Johnston, Ruth L. Stringer and David Santillo

Chemical mixtures, the problem defined

Ecotoxicology, as a discipline, continues to mature. It is clear that the study of the interaction of toxicants with natural ecosystems has moved considerably beyond simple idealised approaches based upon LC_{50} and EC_{50} determinations carried out on test organisms. The development of evaluative end-points based upon a spectrum of responses ranging from sub-organismal to whole ecosystem in scope has created powerful and sensitive tools for the detection of deleterious environmental effects. Yet, as a recent comprehensive discussion of ecotoxicology within the context of ecological risk assessment has made clear [1], most assessment endpoints still relate to the study of single chemicals. Moreover, tests tend to be conducted using single species of test organisms, or at best highly simplistic organismal communities. This situation places potentially severe limitations upon the application of ecotoxicological techniques in the regulatory arena since chemicals in the environment are generally present in mixtures and impact upon complex multi-species systems.

Indeed, environmental contamination with individual chemicals is the exception rather than the rule. Oil, for example, even when divided into refined fractions, is a highly complex mixture. Sewage and industrial effluents may also be highly complex in chemical composition. In addition, variable determinants of the fate of mobilised chemicals means that chemical complexity is inevitable in environmental media receiving a multiplicity of chemical inputs. The scope of the problem can be gauged from estimates that some 63 000 chemicals are in common use worldwide, that about 3000 account for 90% of total global production and that anywhere between 200-1000 new synthetic chemicals enter the market each year [2]. Other figures suggest that in the EC alone, 50 000 substances are in use of which 4500 are demonstrably toxic, persistent in the environment and have the potential to bioaccumulate [3]. Even in terms of defining priority pollutants according to the three basic properties above, progress has been painfully slow. For example, from the priority candidate list of 129 chemicals selected by the EC from an original subgroup of 500, only 18 have been classified on the basis of daughter directives as EC List I chemicals under EC Directive 76/464/EEC [3] regulating discharges of dangerous substances to the aquatic environment.

On the regulatory front, the 'substance-by-substance' approach, generally applied retrospectively to chemicals which have been positively identified as harmful in the environment, is somewhat at variance with the need for prospective regulation of the mixture of chemicals released to the environment. At the same time, while great progress has been made in defining biomarkers of toxic effects [4], these tend to diminish in utility as indicators of causality with increasing complexity of the system under consideration [5]. In other words, while a sub-organismal or organismal biomarker may well allow identification of an effect at the population or ecosystem level, the precise cause of the effect is unlikely to be readily identified. This leaves ecotoxicology on the horns of a dilemma. Should work be focussed upon identification of a range of generalised stress biomarkers, with reliance on other means to establish causality? Or should a battery of chemical-specific responses be sought which allow rapid identification of the causal agent in any one situation?

In practice, both types of biomarker specified above are likely to be developed further in the future, although indicators at community level may increasingly be favoured over chemical-specific varieties. Indeed, chemical-specific biomarkers such as imposex in whelks exposed to tributyltin, are likely to remain rare.

The utility of community-level biomonitoring has recently been demonstrated by a powerful study of the environmental effects of oil and gas exploration in Norwegian waters [6] which, nonetheless, cannot define causal links. Using multivariate statistical analysis, the authors detected subtle yet significant alterations to the benthic community at distances of several km from individual drilling installations and over areas of up to 100 km^2 surrounding an oil field. Previous studies, based on standard toxicity tests, had greatly underestimated the extent to which discharges of drill cuttings and produced waters impact on the surrounding ecology. However, while multivariate techniques may be more sensitive to low levels of environmental stresses, they provide only limited insight into the causal factors and their modes of action in the community. Discharges from offshore installations, as from other sectors, are highly complex, containing a huge range of organic and inorganic components [7]. Close covariance of their concentrations over the affected area renders the identification of components primarily responsible for the observed effects extremely difficult or impossible.

Hence, ecotoxicological studies at high levels of organisation may well develop into the tool of choice for detecting deleterious effects upon natural systems, or conversely, for following progressive improvements in environmental quality. It is questionable, however, whether a robust regulatory framework can be devised for the universe of chemicals introduced into the environment from anthropogenic sources on the basis of such ecotoxicological studies alone. This article considers the regulatory problems posed in this regard by complex mixtures of chemicals. It

Paul A. Johnston, Ruth L. Stringer and David Santillo, *are at the Greenpeace Research Laboratories, University of Exeter, North Park Road, Exeter, EX4 4QE, UK.*

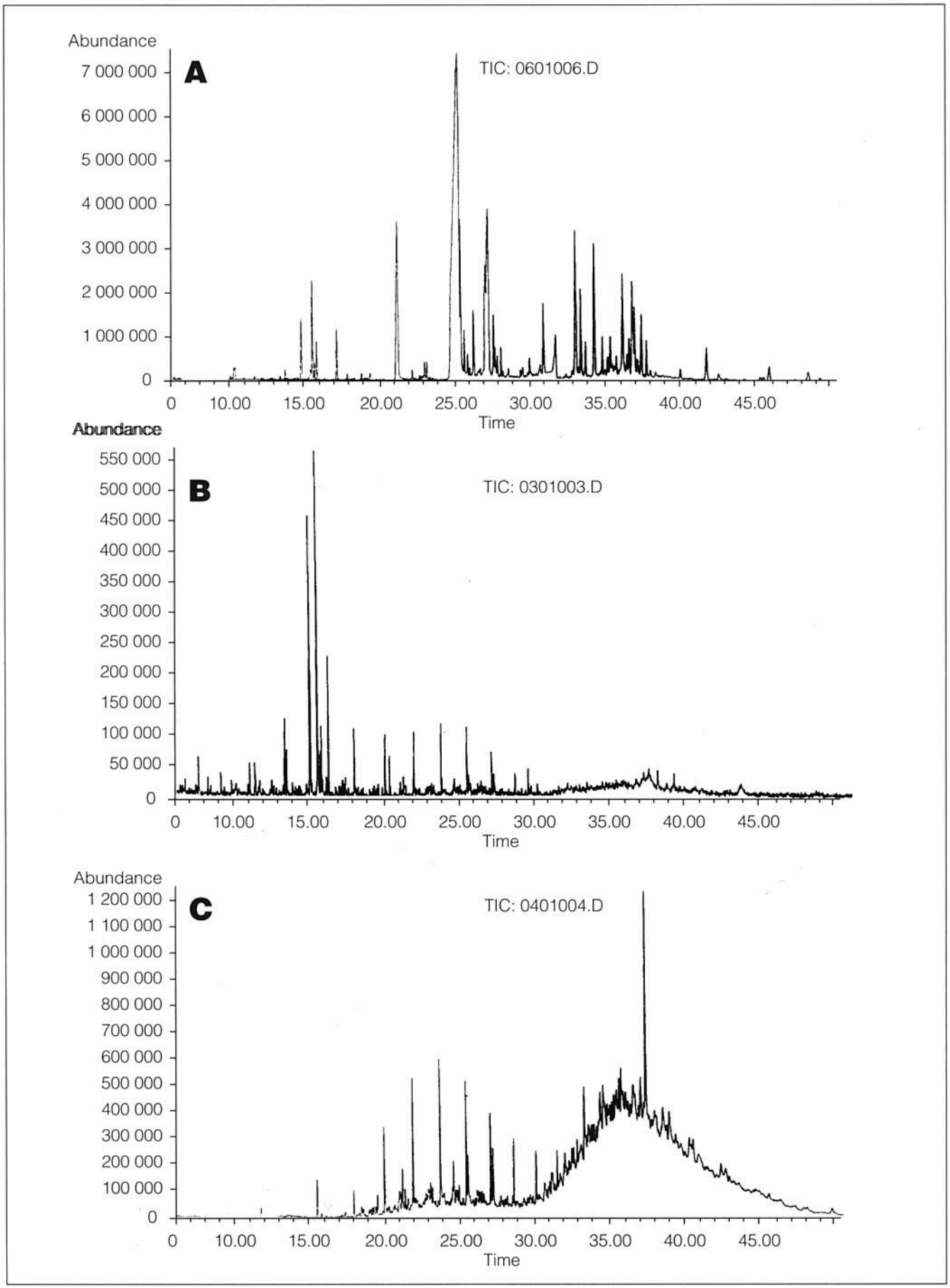

Figure 1. *Traces obtained from solvent extracts of effluent analysed by gas chromatography/mass spectrometry. Each peak on these traces represents an individual chemical compound. Attempts to identify compounds from similar traces using computer based probability matching methods produce data as shown in Table 1. Methods are described in [9]. Traces are (A) an industrial effluent, (B) a combined sewage and industrial effluent, (C) a sewage effluent.*

Table 1. Number of peaks resolved under identical conditions of sample preparation and machine settings from simple hexane extracts of effluent samples. Samples were unmodified effluents as discharged. Peaks matched against the WILEY spectral library at >90% probability and <90% but >50% probability levels are recorded with the percentage remaining unmatched at each probability in brackets.

Sample	Industrial sector	Peaks	Matched > 90% (% unmatched)	Matched < 90% > 50% (% unmatched)	Location
A	Petrochemicals	30	8 (73.3)	15 (23.3)	Italy
B	Mixed sewage/industrial	18	4 (77.7)	10 (22.2)	Italy
C	Aluminium	16	5 (68.8)	11 (0.0)	Italy
D	Mixed sewage/industrial	132	23(82.5)	50 (44.7)	UK
E	Industrial	158	36 (77.3)	79 (13.5)	UK
F	Industrial	16	9 (43.75)	4 (18.75)	Spain
G	Sewage	26	2 (92.3)	12 (46.15)	France

argues that a fundamental reappraisal of regulatory mechanisms is needed if ecotoxicology is to develop to full potential in the protection of natural ecosystems.

Characterisation of complex effluents

Figure 1 shows traces obtained from the analysis of liquid/liquid extracts of three effluents. In each case, identical machine parameters were used and the analysis was conducted using a gas chromatograph with mass selective detection in scan mode (GC-MS). Each of the peaks recorded in the analysis represents a single chemical compound, assuming that no co-elution of substances has taken place. Modern GC-MS methods allow the automated identification of components of chemical mixtures making the technique extremely useful in environmental studies. Nonetheless there are important limitations. Initial screening of an effluent solvent extract may isolate 150 compounds (see Table 1). The recovery of a given analyte will depend upon its properties and the extractive conditions. Hence, solvent extraction as used in these examples will favour the extraction of non-polar compounds. Polar compounds present will require modified extraction procedures and the application of an alternative chromatographic process for example liquid chromatography in conjunction with mass spectrometry (LC-MS).

Moreover, common environmental pollutants, particularly oil, can interfere substantially with the isolation of minor components. Application of sequential clean-up techniques is widely practised and the methods are tailored to removal of all but the specific target compounds. Some of these clean-up techniques are highly labour and cost intensive. Examples include those used for the PCBs and organochlorine pesticides or for the chlorinated dioxins and dibenzofurans [8]. Hence, attempts to improve resolution will generally result in the loss of analytical information, implying that full characterisation will be a multi-step as well as a multi-technique process.

Identification of the various components present in a mixture is also problematical. Table 1 shows the result of attempting to match experimentally-derived mass spectral traces with the 136 000 specimen traces held in the WILEY library. An identification recorded at a probability of 90% or greater is regarded as robust although it may not be possible to distinguish between positional isomers of a chemical on the basis of GC-MS alone. Below 90% probability but above 50%, identification is regarded as tentative, while below 50% a compound is regarded as unidentified. Using these practical, but by no means infallible categories, it is found that a high percentage of chemicals isolated from effluents remains unidentified. In general it is also found that the more complex the effluent, the higher the proportion of chemicals which remains unidentified.

There are a number of contributory factors. In complex effluents, analytical interferences are more likely. The probability-based matching techniques are also less than perfect. Essentially one imperfect set of data is compared with another imperfect set using an imperfect algorithm. Different spectra are obtained from different types of mass spectrometers. Most of the spectra in commercially available libraries were originally obtained from magnetic sector machines and some manufacturers modify the spectra in an attempt to compensate for this. In addition, original data are already known to be partly corrupted in some cases due to artefacts and impurities in the reference compounds processed. Hence, using automated GC-MS techniques, there is a high potential for false positive or false negative identifications [9].

Currently, therefore, the analyst must use a certain degree of professional judgment in interpreting library search results in order to avoid misreporting and it is unlikely that this subjective element will ever entirely disappear from such work. More critically, it is unlikely that library compilers will catch up with the five million or so known organic chemical structures or with the new chemicals brought to the market each year together with their associated contaminants, by-products and degradation residues. This has far-reaching implications for toxicological studies. If it is not possible to identify a compound present in a discharge, then it is impossible to assess the toxicological implications of its discharge even using standard testing methodologies. It follows that ecological risk assessment frameworks will similarly fail.

Some indication of the difficulties which may be experienced is evident from the elegant study conducted on unknown compounds found in shellfish sampled in the River Mersey (UK) [10]. Chromatographic analysis of tissue homogenate extracts from intertidal mussels revealed a large

Table 2. Compounds isolated and identified to a probability greater than 90% using solvent extraction and GC-MS from an effluent discharging to the Mersey Estuary via a sewage treatment works with primary treatment. In all 111 compounds were isolated and hence reasonable identification was achieved for 42 of these ie 37%.

Benzene, 2,4-dichloro-1-methyl-
Benzenamine, 2,5-dichloro-4-nitro-
Benzene, chloro-
Benzene, 1,3-dichloro-2-methyl-
Benzene, 1,3-dichloro-
Benzene, 1,2-dimethyl-
Benzene, methyl-
Benzene, 1-chloro-4-methyl-
Thiophene, 3-butyltetrahydro-2-methyl-
Methanone, diphenyl-
Benzene, ethyl-
Benzene, 1-chloro-2-methyl-
Benzene, 1,1′-methylenebis-
1-Bromo-1,1,2,2,2-pentafluoroethane
1,1′-Biphenyl, 2-phenoxy-
Benzoic acid, methyl ester
Benzoic acid, ethyl ester
Benzene, (chloromethyl)-
Benzene
2,4-Hexadiyne
1,5-Hexadien-3-yne
1,3-Hexadien-5-yne
Methanone, (4-chlorophenyl)phenyl-
Benzene, 1,1′,1′′-methylidynetris-
Methanone, (2-methylphenyl)phenyl-
Benzene, 1-isocyano-2-methyl-
Benzene, 1-methyl-4-(phenylmethyl)-
Benzeneacetonitrile, .alpha.-(phenylmethylene)-
Benzyl benzoate
1-Propanone, 1,3,3-triphenyl-
9,10-Dihydroanthracene
Benzene, 1-methyl-2-(phenylmethyl)-
Benzeneacetonitrile, .alpha.-phenyl-
Benzophenone, 3′-methyl-
Benzaldehyde
Methanone, diphenyl-, (diphenylmethylene) hydrazone
1,1′-Benzene, 1,1′-[thiobis(methylene)]bis
4-ethylphenyl acetate
Benzeneacetic acid, methyl ester
Benzenemethanethiol
m-Benzyldiphenylmethane

number of unidentified peaks, including 13 with retention times similar to hexachlorocyclohexane (HCH). These did not correspond to any known isomers of HCH and commercially available libraries of mass-spectra yielded matches of insufficient quality to allow reliable identification. Further analysis, resorting to NMR techniques, identified the most prominent peak as a methyl- HCH; the authors speculate that the other peaks represent isomers of this compound or higher-chlorinated homologues. The relatively high abundance of these compounds compared to that of HCH, with their occurrence apparently unique to the Mersey estuary, is indicative of a highly significant local source. This is only one of a number of organohalogen compounds routinely discharged to the Mersey. Analytical uncertainties are inevitable given the numbers of chlorinated organic chemicals which can be isolated from a single discharge and the number of chemicals which cannot be readily identified (Table 2; Figure 1 a). The situation may be further complicated by side reactions taking place between the components of the effluent.

However, even if the identifications are reliable and the sources of the compounds can be traced and the discharges fully characterised, any hazard assessment is likely to be fatally compromised by the lack of toxicological methods to assay the potential impact of these discharges, and to regulate on this basis.

Toxicology of mixtures

Toxicological test approaches to mixtures have progressed little in over four decades, although undoubtedly testing regimes have improved in terms of the degree of control exerted over variables. The basic classification for joint toxic action dates back to the early 1950s, evolved only slightly through to the 1970s and appears in modern texts on the subject [1]. The potential for joint toxic action is most commonly represented in diagrammatic form by isobolograms similar to Figure 2. Isoboles are lines representing equal effect. Those falling into areas of more-than-additive action or antagonistic action indicate toxic interactions between chemicals. There are no standard biometrical models which fit these cases. Moreover, there has been no progress in incorporating more than two chemicals into the evaluation which is perhaps understandable given the vast number of response permutations which could then occur.

Overall, this has led to relatively simple approaches being adopted in the toxicological testing of mixtures. In the case of a complex effluent, testing of the whole effluent may be carried out and this is increasingly being developed for use in regulation, control and assessment procedures. Even if these tests indicate toxic action in an effluent, they provide no insight into the toxic component involved. If chemical analysis reveals certain compounds present at levels above their known effective concentration then this may provide the basis for ameliorating the effect. Alternatively, the effluent will need to be fractionated, the individual fractions tested and the most toxic fractions refractionated and chemically analysed until the culprit chemical(s) are isolated.

The methodological and other limitations are amply illustrated by some practical examples. In a study of the chemicals present in the surface water of UK estuaries [11], 71 industrially sourced chemicals were found. These were restricted by sample preparation to those of low to medium polarity and

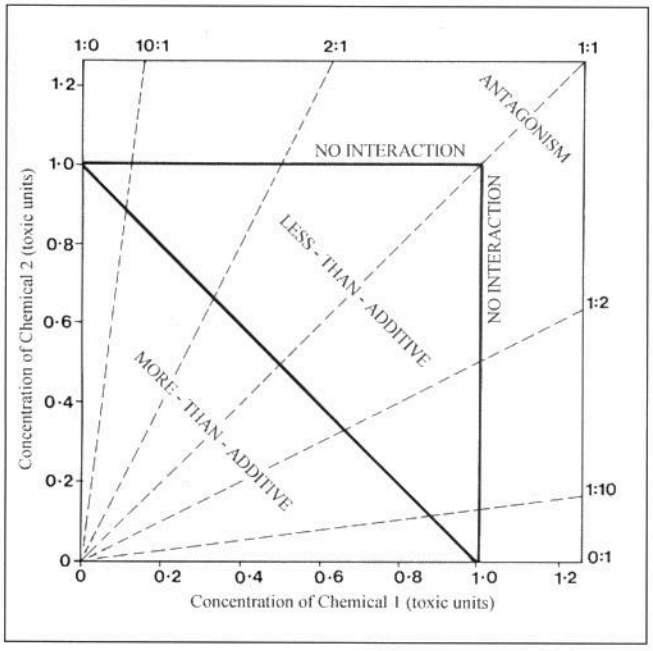

Figure 2. *Isobologram showing lines of equal response to a mixture of toxic chemicals using LC50 or EC50 as a test endpoint. The solid lines indicate the set of concentrations of Chemicals 1 & 2 at which the response occurs and indicate the potential toxic interactions. The broken lines denote fixed concentration ratios of the two chemicals. No standard biometric methods exist for combinations which are more than additive or which are antagonistic.*

hence represent only a proportion of the total number probably present. Of these, following a search for toxicity data and application of quantitative structure activity relationship (QSAR) techniques, an estimate could be made of the toxicity of only 68% of the total. Although none of the chemicals was considered to be present at levels hazardous to aquatic life, oyster embryo larvae used to conduct *in situ* toxicity tests revealed that in the case of some estuaries, toxic effects were evident. Although this study concluded that the observed effects were due to the additivity of the chemicals, there is little empirical basis for this conclusion, since the study protocol would have been unable to detect departures from additivity. It is essentially a general statement based on collected toxicological observations rather than an established principle. More importantly, while this study conceded that toxicological phenomena in the environment may be due to the joint action of chemicals, it also concluded that accurate prediction of the joint effects of complex mixtures of substances is not possible at present.

Analytical fractionation has been used in attempts to identify the hormonally active chemicals present in sewage effluents. The chemical disruption of endocrine systems has been attracting increasing attention in recent years [12,13]. The identification of hormonal activity in sewage effluents resulting in feminisation of male fish has provoked a large-scale study to identify the active components [14]. Liquid chromatography has been used to fractionate sewage effluents and has isolated a 'hot fraction' accounting for 80% of the oestrogenic activity present in these effluents [15]. This fraction is itself a mixture of chemicals and will require further work to characterise the specific agents responsible. Even to get to this stage, over 1500 separate fractions have been assayed, each at various concentrations, a not inconsiderable workload and one with clear resource implications.

The examples outlined represent opposite sides of the coin. In the case of the first study, a set of identified chemicals have been associated with a toxic effect based upon their joint activity. The nature of the joint activity has not been explicitly defined. In the case of the second study, an identified effect has been associated with a mixture of as yet unidentified chemicals. In each case, the implied workload in teasing out the precise relationships is likely to be highly cost intensive and will possibly not fully resolve the issues. There is a further important situation involving chemical mixtures which has recently been identified, where a mixture of chemicals producing an effect causes marked departures from the classical dose–response relationships. A recent study [16] investigated the effect of exposure to contaminated sediments upon indices of the cellular defence capability of crustaceans.

The experiment used test conditions involving 100% clean sand, 100% contaminated sediment and an intermediate mixture of 95% clean sand and 5% contaminated sediment. The experiment was set up to mimic conditions in much larger mesocosms designed to explore the relationship between sediment contamination and fish disease. Unsurprisingly, perhaps, indices of immune function were perturbed by exposure of the test animals to the 100% contaminated sediment, but were more severely affected by the intermediate mixture. Tank effects could be ruled out due to similar findings with replicate systems. Similar observations involving a departure from monotonic responses have been made in sediment LC_{50} assays conducted in the United States. Hence, this is not an isolated finding and brings into the equation the possibility of changes in the biological and/or physico-chemical properties of diluted sediments. The phenomenon is highly significant since it violates the statistical assumptions used in conventional toxicity test procedures and subsequent processing of data. A U-shaped dose–response curve, moreover, implies that environmental impacts of sediments contaminated with a mixture of chemicals could be significantly increased by their mobilisation into cleaner systems.

The regulatory perspective

From the regulatory perspective, the foregoing observations serve to define, but not resolve, a considerable conundrum. On the one hand increasingly sensitive biomarkers and community-based techniques are becoming widely applied to the detection of environmental effects but are of limited use in defining causality. Analytical techniques, although sophisticated, cannot routinely fully characterise complex effluents. Toxicological information on chemicals identified by the application of these analytical techniques is often scant. Moreover, existing toxicological procedures cannot be used to evaluate chemical mixtures in a robust manner. Indeed when applied to mixtures they may define hazard to an extent but not reveal the causal factors without intensive further analytical work. Matrix effects may further reduce the utility of test procedures by causing departures from classical dose–response relationships.

While all of the techniques, applied singly or together, therefore, have a role in evaluating environmental impact, they are not likely, in the context of chemical mixtures, to provide the numerical framework generally required by

legislators formulating regulatory regimes. More simply, greater understanding of natural systems and their responses does not *per se* make application of this knowledge effective in a regulatory sense. This leaves evaluation of chemicals on a 'substance-by-substance' basis as the default *modus operandi*.

The logical way forward through the apparent impasse is often dismissed as naive, simplistic or simply impossible. Zero-emission, however, is a goal whereby regulations represent resting places on the way to achieving it [17]. Already, there are signs that international regulators are beginning to recognise the limitations of current approaches. The 'Virtual Elimination' strategy espoused by the International Joint Commission on the Great Lakes is essentially a zero discharge strategy [18]. More recently the Fourth Ministerial Conference on the Protection of the North Sea [19] committed signatory states to the cessation of all discharges, emissions and losses of hazardous substances within 25 years. Again, this is essentially a move towards zero-emissions.

This is all good news for ecotoxicologists since they can hope in the future to find ecotoxicology increasingly evolving as a prospective discipline. This will prove a considerable contrast to the retrospective analyses which currently dominate the field. In addition, by freeing the discipline progressively from the needs of regulators, it will be in a better position to come to terms with the inherent uncertainties and areas which are currently poorly understood. The ecotoxicological study of complex mixtures of chemicals is undoubtedly a daunting area of study, but one which is fundamental if the discipline is to have a meaningful application in the real world.

References

1. Suter, G.W. (1993) (ed.) *Ecological Risk Assessment* (Lewis Publishers, Boca Raton).
2. Shane, B.S. (1994) Introduction to ecotoxicology. In *Basic Environmental Toxicology*, L.G. Cockerham and B.S. Shane, eds (CRC Press Boca Raton), pp. 3–10.
3. Edwards, P. (1992) *Dangerous Substances in Water: A practical guide*, (Environmental Data Services Ltd, London).
4. Depledge, M.H. and Fossi, M.C. (1994) The role of biomarkers in environmental assessment. *Ecotoxicology*, **3**, 161–172.
5. Suter, G.W. (1994) Endpoints of interest at different levels of biological organisation. In *Ecological Toxicity Testing: Scale Complexity and Relevance*, J. Cairns and B.R. Niederlehner, eds. (Lewis Publishers, Boca Raton), pp. 35–48.
6. Olsgard, F. and Gray, J.S. (1995) A comprehensive analysis of the effects of offshore oil and gas exploration and production on the benthic communities of the Norwegian continental shelf. *Marine Ecological Progress Series* **122**, 277–306.
7. Hudgins, C.M. (1991) *Chemical Usage in North Sea Oil and Gas Production and Exploration Operations* (Oljeindustriens Landsforening, Stavanger, Norway).
8. Kiceniuk, J.W. and Ray, S. (eds) (1994) *Analysis of Contaminants in Edible Aquatic Resources* (VCH, New York).
9. Johnston, P.A. and Stringer, R.L. (1991) Protecting the North Sea: the analytical challenge. *Analytical Proceedings*, **28**, 249–250.
10. McNeish, A.S., Leah, R.T., Connor, L. and Johnson, M.S. (1994) Methyl-hexachlorocyclohexane in mussels (*Mytilus edulis*) from the Mersey Estuary. *Marine Pollution Bulletin*, **28**, 254–258.
11. Matthiessen, P., Thain, J.E., Law, R.J. and Fileman, T.W. (1993) Attempts to assess the environmental hazard posed by complex mixtures of organic chemicals in UK estuaries. *Marine Pollution Bulletin*, **26**, 90–95.
12. Colborn, T. (1994) The wildlife/human connection: modernizing risk decisions. *Environmental Health Perspectives*, **102**, Supplement 12, 55–59.
13. Danish Environmental Protection Agency (1995) *Male Reproductive Health and Environmental Chemicals with Estrogenic Effects* (Danish Environmental Protection Agency).
14. FWR (1992) *Effects of trace organics of fish. Final Report FR/D 0008* (Foundation for Water Research, Marlow, UK).
15. ENDS (1995) *Search for Mystery Oestrogens. Environmental Data Services Report No. 249, October 1995* (Environmental Data Services, London), pp. 4–5.
16. Smith, V.J., Swindlehurst, R.J., Johnston, P.A. and Vethaak, A.D. (1995) Disturbance of host defence capability in the common shrimp, *Crangon crangon* by exposure to harbour dredge spoils. *Aquatic Toxicology*, **32**, 43-58
17. Sprague, J.B. (1991) Environmentally desirable approaches for regulating effluents from pulp mills. *Water Science and Technology*, **24**, 361–371.
18. IJC (1994) *Seventh Biennial Report on Great Lakes Water Quality* (International Joint Commission on the Great Lakes, Windsor, Ontario).
19 MINDEC (1995) *Ministerial Declaration of the Fourth International Conference on the Protection of the North Sea, Esbjerg, Denmark, 8-9*