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RESEARCH ARTICLE

C. Hoffmann · D. Sales · N. Christofi

Combination ecotoxicity and testing of common chemical discharges to sewer using the *Vibrio fischeri* luminescence bioassay

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Abstract In order to investigate possible synergistic or antagonistic (more or less than additive) toxicity effects, mixtures of chemicals were tested in water using a microbial bioassay. Ten toxicants (3,4-dichloroaniline, 3,5-dichlorophenol, cadmium, chromium, copper, Lindane, linear alkylbenzene sulphonate, pentachlorophenol, toluene, zinc) were chosen on the basis of their common occurrence in industrial effluents within local waste water treatment plants. These toxicants also cover a wide range of modes of toxic action, namely, polar and non-polar narcosis, membrane disruption, respiratory disruption, uncouplers of oxidative phosphorylation, biochemical disruption and enzyme inhibition. Efficient screening for possible combination toxicity between toxicants involved testing the chemicals both singly and in triplet combinations. The triplets were based on four replicates of a balanced incomplete block design (BIB). A standardised *Vibrio fischeri* rapid toxicity bioluminescence assay was used. The combinations tested showed that only one mixture was found to be significantly more toxic than expected from the pure single-toxicant results. Two triplets were significantly less toxic. Further tests on the more toxic triplet showed that the effect was due to only one of the 45 pairs originally screened. It is concluded that synergistic effects in combinations of toxicants are rather rare in bioluminescence systems utilising common effluents discharged to sewer.

Keywords *Vibrio fischeri* · Luminescence assay · Synergism · Antagonism · Combination ecotoxicology

C. Hoffmann · D. Sales · N. Christofi (✉)
Pollution Research Unit, School of Life Sciences,
Napier University, 10 Colinton Road, Edinburgh,
Scotland EH10 5DT, UK
E-mail: n.christofi@napier.ac.uk
Tel.: +44-131-4552490
Fax: +44-131-4552291

Introduction

Many biological systems show a dose response in the presence of a toxicant. This often takes the form of a decline from initial levels to zero with increasing concentration of the toxicant. Examples relevant to effluent testing include *Vibrio fischeri* luminescence, nitrification inhibition and enzyme inhibition bioassays [8]. Some biological responses may show a decline to non-zero levels, e.g. ATP activity [7]. The non-zero levels typically vary between toxicants, which introduces an extra level of complexity. Systems may show an enhanced (but usually relatively small) response (hormesis) at low levels of some toxicants [2, 6].

Much of ecotoxicity testing is concerned with the establishment of defined endpoints, such as the IC_{50}, for particular singular toxicants. However, single toxicants are seldom found outside the testing environment of a laboratory, and aqueous matrices including potable water are complex mixtures of many chemicals [12]. It is usual for such mixtures to be discharged into the environment.

There are several ways to classify the actions of mixtures of toxicants. Additive effects of a mixture of toxic chemicals are those that manifest in a summation of the toxicities of the individual constituents [10]. Although the term synergism appears in the literature, it does not appear to have been clearly defined. It may be important to determine responses that can occur in the absence of any interactions between toxicants. Synergism may then be clearly defined as a more toxic response that is outside this range of possible responses. Synergism has been defined as the interaction of one or more chemicals to produce an effect greater than the sum of the two agents acting separately (more than additive). Antagonism embraces the effects of two or more agents such that the action of any one of them on a response is reduced (less than additive).

Different applications ask different questions of toxicity tests. In an industry in which a waste stream is to be

investigated, the question may relate to the quantity of solvent, for example, that can be allowed to be discharged to sewer under a given consent. The solvent in the waste stream, however, will interact with an unknown number of compounds in the sewerage system and there is a need to know whether the combinations of compounds will enhance the initial toxicity of the solvent.

Parrot and Sprague [21] found that copper and zinc acted in an additive fashion when tested with *V. fischeri*. Their hypothesis was that toxicants with similar types of action would have an additive effect, and that combinations of toxicants with different types of action would have a less-than-additive effect. Pedersen and Petersen [22] reported results obtained by comparing the variability of species sensitivity (in their study *V. fischeri*) to complex mixtures. They showed that the joint toxicity of the complex mixtures could be described by assuming that the toxicity of the individual substances in the mixtures was more or less additive. This assumes, however, that the toxicity of all pure toxicants of a mixture is known. In a recent study on phenolic wastes from a chemical plant, it was concluded that the toxicity of the whole effluent could be accounted for on the basis of the individual constituents but that the toxicities were only partially additive [13].

An ecotoxicity method design must include robust statistical analysis to be meaningful. Statistical design is based on three basic concepts: randomisation, replication and local control [3]. Randomisation of experimental units to treatments provides a safeguard against potential bias. Replication is necessary to analyse variation, and local control (or blocking) is a tool to remove unwanted variation [3]. These criteria are rarely, if ever, met in ecosystem studies. However, the advantage of the *V. fischeri* test system is that it is well controlled locally, and that both randomisation and replication can easily be achieved with the correct experimental design.

Here we present a set of experiments that were designed in a mathematically and biologically rigorous method to investigate the occurrence or absence of synergism. The standard *V. fischeri* luminescence toxicity bioassay procedure is used and a set number of toxicants are tested in a predetermined grid to test for possible interactions. The aim is to allow the design and execution of experiments that are able to show combination effects between toxicants commonly discharged to sewer.

Materials and methods

Toxicity test method

Toxicity was determined according to DIN 38412 method B [9], utilising the luminescent bacterium *V. fischeri* as the test organism. The tests were not pH controlled but pH was monitored to detect deviations from acceptable values for the test. Both the 5- and 30-min IC_{20} and IC_{50} values were established with the bioassay for single toxicants. The toxicants used were 3,4-dichloroaniline (3,4-DCA), 3,5-dichlorophenol (3,5-DCP), cadmium, chromium, copper, Lindane, linear alkylbenzene sulphonate (LAS), pentachlorophenol (PCP), toluene and zinc. For those compounds that had low water solubility or insolubility (Lindane, PCP, toluene, 3,4DCA), acetone was used as a co-solvent. Initially, the IC_{50} for acetone in *V. fischeri* was determined (17,343 mg/l with a "no observable effect concentration"). The final concentration of acetone used in a toxicant mixture was less than the NOEC. Acetone is highly volatile so care was taken to decant it from its container only when ready to use in the test. During testing, Lindane was still observed to precipitate during the tests. All toxicants were prepared using Good Laboratory Practice and chemical analyses of individual toxicants was carried out by Scottish Water Analytical Services (NAMAS-accredited laboratory) on solutions to ensure correctness of concentrations. Mixtures of toxicants were not analysed.

There were two dilution steps inherent in the experimental method of the bioassay. Since all toxicants were mixed with two others as a triplet in a 1:1:1 ratio, the individual toxicant was effectively diluted 1:3. A further 1:2 dilution occurred when the toxicant mixture was combined (1:1) with the *V. fischeri* test organism. Together this represented a 1:6 dilution of the original concentration. To achieve a concentration above the 30-min IC_{20} in the first test cuvette, the initial concentration had to be at least six times the 30-min IC_{20} value for each individual toxicant. For all toxicants, solutions with a six-fold concentration of the 30-min IC_{20} mg/l were prepared and were kept in a refrigerator at 3 ± 3 °C.

Analysis of pairs of toxicants using a balanced incomplete block design

The interaction between pairs of toxicants was investigated. To test all 36 possible pairs of the nine toxicants was onerous. To rationalise the work plan, it was decided that the pairs would be investigated as parts of triplets. An experimental set-up based on binomial coefficients was designed in which the method of choosing pairs relied on Eq, 1:

$$\binom{n}{r} = \frac{n!}{r!(n-r)!} \tag{1}$$

where n = number of items and r = number of pairs. For nine toxicants arranged in pairs, Eq. 2 follows:

$$\binom{9}{2} = \frac{9X8X7X6X5X4X3X2X1}{2X1X7X6X5X4X3X2X1} = 36 \tag{2}$$

These 36 pairs were arranged in 12 triplets in a balanced incomplete block (BIB) design so that each pair occurred once and each toxicant was combined with every other once. The toxicants were then randomised and the list of mixtures thus achieved is given in Table 1 (mixtures 1–12). For each triplet, a dilution series of four relevant concentrations was prepared (two concentrations above and two below the predicted IC_{50} if possible), with a control blank. Each mixture was tested four times to allow an estimate of variation. To prepare for the *V. fischeri* assay, 0.83 ml of each toxicant in a triplet was added to 0.25 ml of 22% NaCl in a cuvette. Three dilution steps of 1:2 were then prepared.

Copper was added to the test protocol (Table 1; mixtures 13–20). The simplest way to achieve this with the minimum number of additional tests was to substitute copper for one of the original nine toxicants, in this case Lindane.

Results

All new toxicant solutions with a concentration of 6× 30-min IC_{20} mg/l were tested for their IC_{50} after 5 and 30 min to test whether the diluted reagent used for the triplets experiments behaved as expected. The results are presented in Table 2 and are compared with the IC_{50}

Table 1 Composition of triplet mixtures 1–12 used in synergism study. *PCP* Pentachlorophenol, *3,5 DCP* 3,5-dichlorophenol, *LAS* linear alkylbenzene sulphonate, *3,4 DCA* 3,4-dichloroaniline, *Lindane* 1,2,3,4,5,6 γ-hexachlorocyclo-hexane

Mix	Chemical constituents		
1	Cadmium	PCP	Chromium
2	Cadmium	3,5 DCP	Lindane
3	Cadmium	Zinc	Toluene
4	Cadmium	LAS	3,4 DCA
5	PCP	3,5 DCP	Zinc
6	PCP	Lindane	LAS
7	PCP	Toluene	3,4 DCA
8	Chromium	3,5 DCP	3,4 DCA
9	Chromium	Lindane	Toluene
10	Chromium	Zinc	LAS
11	3,5 DCP	Toluene	LAS
12	Lindane	Zinc	3,4 DCA
13	Cadmium	3,5-DCP	Copper
14	PCP	Copper	LAS
15	Chromium	Copper	Toluene
16	Copper	Zinc	3,4-DCA
17	Copper	Lindane	Zinc
18	Copper	Lindane	3,4-DCA
19	Copper	Lindane	LAS
20	Copper	Lindane	Toluene

Table 2 Comparison of the 30-minIC_{50} for the single toxicant concentrations used in thetriplet mixtures in mg/l ($n=1$) and the 30-minIC_{50} for the same toxicants established in the DTOX Project [5]

Toxicant	This study 30-min IC_{50}	DTOX Project 30-min IC_{50}	*n*
Cd (as $CdCl_2$)	5.85	7.59	8
Cr (as K_2Cr2O7)	21.79	18.14	35
Cu (as $CuCl_2$)	0.26	0.22	10
3,4-DCA	0.50	0.72	6
3,5-DCP	3.18	3.19	26
LAS	20.75	19.40	9
Lindane (γHCH)	14.13	19.13	5
Toluene	No effect	20.75	6
PCP	1.13	1.40	4
Zn (as $ZnSO_4$)	0.44	0.46	35

values from a previous study [5]. Each test was only carried out once as it was intended to act as a safeguard to check that comparable IC_{50} values were produced. This was the case for all toxicants but toluene.

Four replicates for each of the 16 mixtures plus ten individual IC_{50} tests were conducted. All results from testing the triplet mixtures were recorded as percentage (%) concentration (Table 3). It was assumed that 50% was equal to the IC_{50} value. Thus, the lower the value, the more toxic the mixture. Often a mixture was not toxic to the *V. fischeri* assay, resulting in the lower number of degrees of freedom (*n*; Table 3).

All mean 30-min IC_{50} were plotted and standard deviations calculated where possible (Fig. 1). For mixtures in which no calculable toxicity was found, or only one or two toxic results were obtained, it was not possible to calculate a standard deviation. Ten mixtures were found to have a toxicity higher than 50% (mixtures 1, 2, 3, 4, 5, 6, 7, 8, 11, 13) and five were less toxic than

Table 3 Mean 30-min IC_{50} for each toxicant mixture ($n=4$)[a]. *n/a* Not appropriate due to number of samples

Mix	Mean 30-min IC_{50} (%concentration)	*n*	Standard deviation (% concentration)
1	22.9	4	6.8
2	27.6	3	15.4
3	44.4	4	11.4
4	27.0	4	2.73
5	9.22	3	7.90
6	26.2	3	8.02
7	30.5	4	5.46
8	22.9	4	1.40
9	118.6	3	29.5
10	74.6	3	52.4
11	39.6	2	n/a
12	120.7	4	13.9
13	44.5	1	n/a
14	108.7	4	4.08
15	170.5	4	16.0
16	89.6	3	8.55
17	not toxic	4	n/a
18	58.5	1	n/a
19	Not toxic	4	n/a
20	Not toxic	4	n/a

[a]In cases in which $n<4$, four tests were actually carried outbut some had a 'non-toxic' result. Those mixtures in which $n<4$ have a biased result, in reality being less toxic

50% (mixtures 9, 12, 14, 15, 16) with two mixtures around the 50% level (mixtures 10 and 18). Mixtures 17, 19 and 20 were not toxic.

Discussion

Models of toxic response

The standard models for analyzing the response to a single toxicant can be thought of as taking a suitably transformed response variable and fitting a weighted regression on the log (concentration) of the toxicant. The usual transformations used are either the logit or the probit of the proportionate reduction from the initial levels. These correspond to the assumption that the tolerance of the organisms involved follows either a logistic distribution or a normal distribution, respectively. These two distributions have very similar shapes, so there is little practical difference between them. The logit transformation [1] is mathematically more convenient and so has tended to replace the more traditional probit (see, e.g. [11]) in recent work.

These models have been widely used in toxicity work and in applications in many other fields. Although not perfect, they do seem to provide adequate models for a wide range of biological and other systems. For the sake of clarity, the discussion will assume that a logit transformation has been used (recently, the use of generalized linear models has been proposed to analyze data without an initial linear transformation [17] and used [23]). The use of probits or the method of fitting the model does not affect the conclusions.

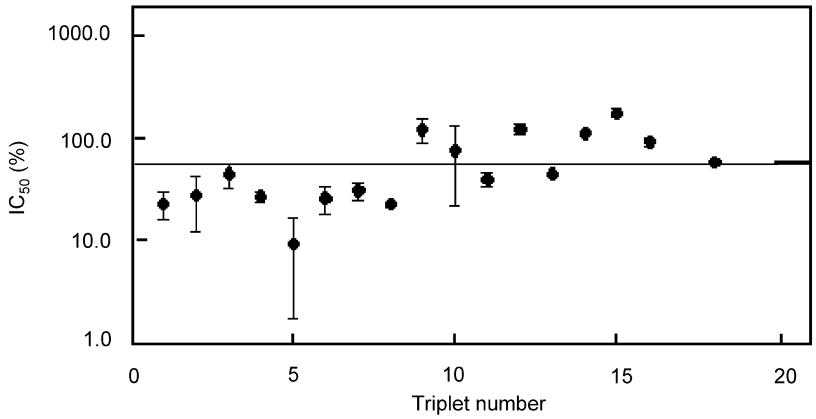

Fig. 1 Mean 30-min IC_{50}(%) with standard deviation for 20 mixtures (mixtures 17, 18 and 20 were not toxic)

In work on toxicity, the slope and intercept from the regression are not always reported. Instead, they are usually used to calculate the toxicant concentration that would give a certain reduction in response. This is typically the EC_{50} (50% effective concentration).

Mixtures of toxicants

The models mentioned apply to a single toxicant. For mixtures of two or more toxicants, it is possible to extend the logit model, but adequate data to fit and test such a model for interactions between toxicants are often not feasible because of practical constraints. The more usual procedure is to form mixtures from known concentrations of pure toxicants and treat each mixture as if it was a novel pure toxicant. The resulting EC_{50} is then expressed as a percentage of the source mixture. This can then be converted back to give the corresponding concentrations of pure toxicants, and these can be compared with the pure EC_{50} or other values [23].

The interpretation of such comparisons is not entirely straightforward. There are two plausible situations that, in general, give different predictions of the expected response to a mixture. These are bioequivalent toxicants and independent toxicants. Real-life situations are anticipated to lie somewhere between these extremes.

Bioequivalent toxicants

If two toxicants affect identical pathways in the same way, then the tolerance distributions will have the same shape but with a shift in the mean. This corresponds to concentrations of one toxicant being a constant multiple of the concentration of the other toxicant. This implies that the slopes of the response curves will be the same. Further, if the EC_{50} concentrations of the pure toxicants and of mixtures are plotted on the same graph, these should all lie on a straight line.

For mixtures of three or more toxicants with similar effects, the same considerations apply. In particular, the EC_{50} of mixtures are expected to lie in a plane. Thus a natural statistic to take for the EC_{50} of a mixture is to divide the concentration of each toxicant by the concentration of the pure EC_{50} and add up these ratios. Values greater than one may indicate a synergistic response and values less than one a reduced response. Note that this statistic only applies to toxicants that are essentially equivalent in their biological effects.

Toxicants with independent effects

If two toxicants act completely independently of each other, then the probability methods can be used to predict the effect of combinations. For example, if the level of one toxicant is at the EC_{20} and the other is at the EC_{40} then the combination would be expected to be at the EC_{52} level. This is because EC_{20} and EC_{40} correspond to 80% and 60% "survival", respectively, giving an overall "survival" of 48%. This is easily extended to more complex mixtures.

In this situation, it is clear that knowledge of the EC_{50} values of the pure toxicants is not sufficient to decide whether there is an enhanced or reduced response; knowledge of the slope parameter in the model is needed as well. Another conclusion from this situation is that even if the logit (or probit) model describes both toxicants perfectly, it will not be a perfect fit to any mixture of them. It is not yet clear how large the departures from the model are likely to be in practice.

Real toxicants

In general, the predicted EC_{50} from independent toxicants will differ from that for equivalent toxicants. As individuals within the population under test are likely to vary in their physiological and viable status, one might expect the tolerance distribution of a toxicant to comprise a general component and a component specific to that toxicant. Thus the tolerance distributions of different toxicants are likely to be positively correlated. The two extreme models considered above correspond to perfect positive correlation and zero correlation, respectively. Thus, there will be a range of EC_{50} values for a mixture that corresponds to an absence of evidence of either an enhanced or a reduced effect.

Table 4 The logit model and examples of the effect of slope on equivalent and independent toxicants

Slope	Equivalent	Independent
−0.5	27.30	9.37
−0.6	14.02	6.45
−0.7	8.71	4.94
−0.8	6.09	4.05
−0.9	4.61	3.47
−1.0	3.69	3.06
−1.1	3.08	2.76
−1.2	2.65	2.54
−1.4	2.09	2.22
−1.6	1.75	2.01
−1.8	1.52	1.86
−2.0	1.36	1.75

Examples using the logit model

To illustrate the differences between equivalent and independent toxicants, an intercept of 2.0 and a range of slope values are chosen. From these values, the EC_{50} of each pure toxicant can be calculated. If two different toxicants had this intercept and the same slope as each other, then it is of interest to consider how a mixture of equal parts of each toxicant would behave. The EC_{50} of the mixture could be determined and the corresponding toxicant concentrations calculated. The two extreme models above can be used to calculate what the toxicant concentrations in the mixture would be. For equivalent toxicants, both will be at half the EC_{50} concentration of the pure toxicant. For independent toxicants, both will be at the 29.3% effective concentration.

Table 4 gives the values of these two concentrations for various slopes. Note that for shallow slopes, two independent toxicants are more toxic than two equivalent toxicants, but the situation is reversed if the slopes are steep. Changing the intercept would change the absolute values in Table 4, but not their ratios. Similar calculations can be carried out for toxicants with different slopes and intercepts, different proportions of toxicants and mixtures of more than two toxicants. They can also be used for different end points, such as the EC_{20}. Thus, if estimates of the intercept and slope of pure toxicants are available, it is possible to obtain upper and lower limits for effective concentrations in arbitrary mixtures of these toxicants.

It is not possible to give an accurate prediction of the effect of two toxicants, even in the absence of any interactions between them. However, upper and lower limits on the response can be calculated. Synergism should be defined to be a more toxic response which is outside these limits.

Combination ecotoxicity using bioluminescence

It is uncommon for the toxicity of mixtures of chemical pollutants to be considered in the assessment of risk to biota in natural waters. Testing of the effects of chemical mixtures discharged to sewer on microbiological processes involved in organic waste treatment (e.g. activated sludge processes) is additionally rare. In order to enhance our understanding of the effects of chemical discharges on waste treatment processes, possible synergistic or antagonistic effects of mixtures of toxicants was investigated using the surrogate bacterium *V. fischeri*. Verhaar et al. [25] defined, on the basis of mode of toxic action, four classes of chemicals. These are nonpolar narcotic (class 1), polar narcotic (class 2), reactive (class 3) and specific (class 4) modes of action [24]. Nine toxicants (3,4-DCA, 3,5-DCP, cadmium, chromium, LAS, Lindane, PCP, toluene, zinc) were chosen on the basis of their relevance (i.e. their regular occurrence in industrial effluents) and covered most modes of toxic action. This included enzyme inhibition afforded by metals, polar (e.g. 3,4-DCA) and non-polar narcosis (e.g. acetone, toluene), membrane disruption (e.g. LAS) and uncoupling of oxidative phosphorylation (e.g. PCP). Acetone was also investigated as it was used as a solvent for a number of toxicants tested.

Initially, copper had not been included in the test protocol because from previous analysis as a single toxicant it was known that copper has an unusually steep reaction curve in the *V. fischeri* assay [15]. This could cause problems in interpreting the results from mixtures. However, Carlson-Ekvall and Morrison [4] reported that the toxicity of copper in raw and digested sewage was much higher than could be explained by the concentration of electrochemically available copper alone. They attributed this to synergism and potentially the formation of lipid-soluble copper complexes. As far as could be gathered from the literature, this was the only evidence of synergism using copper and the *V. fischeri* bioassay, and, it was decided to include copper in the testing of mixtures.

Based on a number of studies of compounds having similar modes of toxic action [4, 18, 22] and on observations made during this project, a hypothesis was made that toxicants generally act independently. According to the multiplicative model of Nash [20], the observed effects of a mixture's components acting alone (expressed as probabilities) are multiplied to predict joint action. Hence, if three concentrations depicting the IC_{20} values were added, the total toxicity should be around 50% of the sample ($0.8\times0.8\times0.8=0.512$). This model classifies mixtures that act according to the prediction of the model as additive, whilst those more or less toxic than predicted are termed synergistic or antagonistic.

There are at least three models that could be used to classify the actions of mixtures together. The relative merits of the concentration-addition model, the multiplicative model, and the isobole diagram method were discussed by the inventors of the latter method, Parrot and Sprague [21]. King [18], using the multiplicative model [20], concluded in a study investigating the toxicities of single and mixed chemicals to respiration, nitrification and *V. fischeri* that synergism was rare.

If the rationale behind the choice for the initial concentration for the mixtures, that toxicants act independently, was true, then an additional effect of toxicity would be observable. After testing the mixtures, only one triplet was found to be significantly more toxic than expected: triplet 5, composed of PCP, 3,5-DCP and zinc. One was found to be significantly less toxic: triplet 15 (chromium, copper and toluene). Two triplets showed a significantly reduced toxicity than expected; these were triplet 9 (chromium, Lindane and toluene) and triplet 12 (Lindane, zinc and 3,4-DCA). It was not clear why that should be so, but it is notable that both triplets contained Lindane and a metal. Lindane is known to be almost water-insoluble, and might have precipitated, thus affecting its bioavailability.

Further work could delineate the toxic constituents through dividing the triplets into their constituent pairs and investigating the interactions in the pairs. Preston et al. [23] used bacteria containing the *lux* reporter gene to examine synergistic, antagonistic and additive effects of metal combinations (pairs). They suggested that the increased toxicity of the metal mixtures may be due to membrane damage by one metal constituent allowing increased influx of toxicants. In our system, it is known that phenolics affect membrane function, and the synergistic effect observed with the Zn/PCP/3,5-DCP mixture could relate to the increased influx of zinc across the cell membrane of *V. fischeri*.

Recently, a mathematical algorithm was used to delineate the toxicity of mixtures of metals [19] and compared to experimental data using the *V. fischeri* assay. Computational calculations were based on additivity as in this study, but in our study mixtures of common organic chemicals and metals discharged to sewer were utilised. Binary and ternary combinations of metals were used and it was shown that additive, synergistic and antagonistic effects are possible with metals combined in equal mass/volume ratios. The interactions could also be demonstrated with varying concentrations of metals in such mixtures. No clear explanations of mechanisms involved could be given for these interactions. In the present study a number of factors may act to give the responses presented, but the data indicate a lack of synergism with common chemicals discharged to sewer as tested using the *V. fischeri* assay. Salinity and pH changes may be implicated in the responses observed, affecting speciation and hence toxicity of metals.

The duration of the test may have affected the responses. In this study, a 30-min incubation was used. Other studies utilise 5 and 15 min and it is accepted that a longer incubation period is necessary to elicit a better response to some metals. With respect to the 30-min incubation period, the results may be affected when using a volatile substance or chemicals that are poorly soluble in water. The use of solvents may not be adequate to maintain solubility in the test system.

The use of the marine *V. fischeri* bacterium does not compromise toxicity assessment as the activated sludge process is well buffered around neutrality and appropriate for the organism. The use of osmotic adjustment is also not a problem as we have shown that the *V. fischeri* assay is as sensitive a test as bioassays utilising chemolithotrophic nitrifying bacteria [16], microbial respiration and enzyme activity [8], and ATP luminescence [7] even when using a sludge matrix for the bioluminescence assay [15].

It must be remembered that, in real water systems, the toxicity of mixtures will depend on the physiological state of the microorganisms and physicochemical factors affecting dissolution, sorption and hence potential uptake of chemicals. Bioavailability of organics (e.g. chlorophenols) and hence toxicity depends on their hydrophobicity and the pH of the environment. The same is true for metals whose solubility is significantly affected by pH. In addition, preferential sorption of chemical constituents will affect toxicity values. Surfactants such as LAS can enhance the solubility of hydrophobic organic pollutants and metals. Such interactions are never fully examined in toxicity bioassays using surrogate test organisms in the laboratory and hence it may be difficult to fully appreciate synergistic, antagonistic and additive effects of toxicant combinations with respect to environmental risk assessment.

Conclusions

Synergistic (more than additive) effects are much debated with regards to industrial discharges and they create anxieties over possible supertoxic compounds that might form in sewers. The main conclusion from these experiments using pollutants commonly occurring in sewers was that such effects are rarely found as determined by the *V. fischeri* test. If it could be proven that these results are indeed a reflection of the real world, this would be encouraging for the purpose of setting discharge consents. It is hoped that strict experimental designs such as this will be carried out with nitrification, respiration, ATP luminescence and other such rapid direct toxicity assessment (DTA) techniques. Then it might be possible to set consent limits for compounds on the basis of their individual IC_{50}—provided these are known.

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