Evaluation of interactive toxic effects of chemicals in water using a *Tetrahymena pyriformis* toxicity screening test

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Abstract

A *Tetrahymena pyriformis* oxygen uptake assay developed for rapid screening purposes was used to investigate toxic effects of combinations of chemicals commonly found in various water environments. Respiratory response was measured over a 5 min period at constant temperature by means of a biological oxygen monitoring system connected to a potentiometric recorder. Mixtures of chemicals produced antagonistic, synergistic, additive or neutral toxic effects, depending on the chemicals used in combination and individual concentrations. Irrespective of the type of combined effect when testing high chemical concentrations, mixtures of low concentrations of these chemicals produced additive effects. Enhancement of the toxic effect when chemicals were mixed indicated that water quality standards based on individual toxicity data do not sufficiently protect the aquatic environment. Biological monitoring can, however, detect such effects and is therefore a very important detection system.

Introduction

The effects of individual toxic chemicals on the aquatic environment have been studied extensively, using various test organisms. When pollution occurs in practice, however, several chemicals are usually present simultaneously. These may interact additively, synergistically or antagonistically, or the observed effect may be due to the toxicity of the dominant constituent.

Much of the work on toxic interaction of aquatic pollutants has been carried out using fish, and involved the development of various models for assessing and predicting combined toxicity (Bliss, 1939; Sprague, 1970; Muska and Weber, 1977). Very few authors have examined the effects of mixtures of toxic chemicals on micro-organisms. Dutka and Kwan (1982) compared four bacterial screening procedures for assessment of the effects of mixtures of toxic chemicals. Combined effects varied from antagonistic or neutral to additive and synergistic. The authors found that each system has its own toxicity sensitivity pattern. There are areas of general concurrence as well as areas of wide divergence in sensitivity. Because of the variety of toxicant concentrations to which biological species react the battery approach using various testing systems is recommended. Interaction of certain metal combinations on algae was examined by Wong and Beaver (1981) and Rai *et al.* (1981). In certain cases the metals acted antagonistically while in others the same metals had markedly synergistic effects. This was also observed by Gray (1974) and Parker (1979) while studying the effect of metal combinations on the growth of ciliate populations.

During recent years a number of rapid microbiological screening tests have been developed, one of which was the *Tetrahymena pyriformis* oxygen uptake bioassay (Slabbert and Morgan, 1982), which provides results within 10 min. Based on physiological functions, these microbiological tests detect low levels of toxicants. In this study the *T. pyriformis* toxicity bioassay was applied to test mixtures of chemicals commonly found in water. The objective of the research was to obtain a better understanding of the modes of action of sub-lethal concentrations of toxicants in mixtures and to evaluate the sensitivity of the system to combined toxicity. Results were used to establish whether water quality standards based upon individual toxicity data adequately protect the microbiota.

Materials and methods

Chemicals

The following chemicals were tested: Hg^{2+} ($HgCl_2$), Cu^{2+} ($CuSO_4$), Zn^{2+} ($ZnSO_4.7H_2O$), Pb^{2+} ($PbCl_2$), Cr^{3+} [$Cr(NO_3)_3$], CN^- (NaCN), F^- (KF), As^{3+} (As_2O_3), BO_3^{3-} (H_3BO_3), NH_3 [$(NH_4)_2SO_4$] and C_6H_5OH. Deionized water was used for the preparation of test solutions and for control tests. Membrane filtered (0,45μm) activated sludge effluent from the Daspoort sewage works near Pretoria (Prinsloo *et al.*, 1978) was used as solvent and control for one series of tests carried out on a mixture of ten chemicals added together at concentrations set as general standard for industrial effluents discharged into receiving waters in South Africa (1962). The chemical composition of the effluent has been reported elsewhere (National Institute for Water Research, 1982). After addition to the *T. pyriformis* cell suspension (20-fold dilution), the concentrations of some of the determinands were as follows: 1,6 mg/ℓ COD (chemical oxygen demand), 0,59 mg/ℓ DOC (dissolved organic carbon), 49,1μg/ℓ MBAS (methylene blue active substances), 2,1 μg/ℓ phenol, 0,13 mg/ℓ NH_3, 31 μg/ℓ BO_3^{3-}, 75,0 μg/ℓ F^-, 1,25 μg/ℓ Cr^{3+}, 1,25 μg/ℓ Cu^{2+}, 4,25 μg/ℓ Zn^{2+}, 1,25 μg/ℓ Pb^{2+} and 2,5 μg/ℓ CN^-.

For purposes of comparison this study also included tests on the individual chemicals making up the various mixtures. Part of the individual toxicity data have already been published in a methodological paper by Slabbert and Morgan (1982).

Test organism

T. pyriformis strain W was cultured axenically at 27°C in a 10 g/ℓ proteose peptone medium (Slabbert and Morgan, 1982). Logarithmic growth phase cells (24 h old) were suspended in Osterhout salt solution (Taylor and Strickland, 1935) by means of gravity filtration before use in toxicity tests. Cells were diluted to a concentration yielding an oxygen uptake rate of approximately 8 units(%)/min.

Toxicity test

The *T. pyriformis* oxygen uptake bioassay was carried out accord-

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ing to the method outlined by Slabbert and Morgan (1982). Oxygen uptake was measured using a biological oxygen monitoring system (stabilized at 27°C) connected to a potentiometric recorder. For each test, 0,25 mℓ of a test solution were introduced into 4,75 mℓ of cell suspension, oxygen uptake rate being recorded continuously before (reference), during (mixing) and after sample addition (test or control). Six replicate tests were carried out on each toxicant and control sample. The effect of test samples on oxygen uptake rate was established 5 min after mixing (exposure period of 5 min). Toxicity results were calculated as percentage inhibition (or stimulation) according to the formula:

TABLE 1
THE EFFECT OF HIGH CONCENTRATIONS* OF SELECTED CHEMICALS ON THE OXYGEN UPTAKE RATE OF *TETRAHYMENA PYRIFORMIS*[α]

Chemical (mg/ℓ)	% Inhibition Individual effect	Combined effect	Type of combined effect
1,0 Hg^{2+} 5,0 Cu^{2+}	57 (±3,4) 22 (±5,9)	78 (±3,1)	ad
5,0 Cu^{2+} 5,0 Zn^{2+}	22 (±5,9) 38 (±4,6)	53 (±7,1)	ad
1,0 Hg^{2+} 5,0 Zn^{2+}	57 (±3,4) 38 (±4,6)	85 (±4,0)	p ad
1,0 Hg^{2+} 5,0 Cu^{2+} 5,0 Zn^{2+}	57 (±3,4) 22 (±5,9) 38 (±4,6)	81 (±1,4)	p ad
1,0 Hg^{2+} 0,5 CN^-	57 (±3,4) 71 (±1,4)	62 (±2,8)	an
5,0 Cu^{2+} 0,5 CN^-	22 (±5,9) 71 (±1,4)	30 (±2,4)	an
5,0 Zn^{2+} 0,5 CN^-	38 (±4,6) 71 (±1,4)	72 (±1,0)	n
1,0 Hg^{2+} 5,0 Cu^{2+} 5,0 Zn^{2+} 0,5 CN^-	57 (±3,4) 22 (±5,9) 38 (±4,6) 71 (±1,4)	46 (±3,1)	an
1,0 Hg^{2+} 500,0 C_6H_5OH	57 (±3,4) 30 (±4,4)	65 (±3,7)	p ad
5,0 Zn^{2+} 500,0 C_6H_5OH	38 (±4,6) 30 (±4,4)	49 (±3,4)	p ad
5,0 Cu^{2+} 500,0 C_6H_5OH	22 (±5,9) 30 (±4,4)	29 (±3,1)	n
0,5 CN^- 500,0 C_6H_5OH	71 (±1,4) 70 (±4,4)	72 (±2,0)	n
1,0 Hg^{2+} 5,0 Cu^{2+} 5,0 Zn^{2+} 0,5 CN^- 500,0 C_6H_5OH	57 (±3,4) 22 (±5,9) 38 (±4,6) 71 (±1,4) 30 (±4,4)	57 (±1,4)	an

*Concentrations exhibiting a marked change in oxygen uptake rate
[α]Effect after 5 min of exposure
Each result is an average of six tests. Standard deviation (S.D.) given in brackets. S.D. for control results ±0,8
ad – additive; an – antagonistic; n – neutral; p ad – partially additive

TABLE 2
THE EFFECT OF LOW CONCENTRATIONS* OF SELECTED CHEMICALS ON THE OXYGEN UPTAKE RATE OF *T. PYRIFORMIS*[α]

Chemical (mg/ℓ)	% Inhibition Individual effect	Combined effect	Type of combined effect
0,5 Hg^{2+} 0,5 Cu^{2+}	5 (±3,4) 3 (±3,2)	4 (±2,0)	n
0,5 Hg^{2+} 0,5 Zn^{2+}	5 (±3,4) 4 (±0,9)	7 (±3,1)	n
0,5 Cu^{2+} 0,5 Zn^{2+}	3 (±3,2) 4 (±0,9)	3 (±2,8)	n
0,1 Hg^{2+} 0,5 Cu^{2+} 0,5 Zn^{2+}	0 3 (±3,2) 4 (±0,9)	5 (±1,4)	n
0,5 Hg^{2+} 1,0 Pb^{2+}	5 (±3,4) 3 (±0,8)[+]	7 (±5,2)	n
0,5 Cu^{2+} 1,0 Pb^{2+}	3 (±3,2) 3 (±0,8)[+]	3 (±1,4)[+]	an
0,5 Zn^{2+} 1,0 Pb^{2+}	4 (±0,9) 3 (±0,8)[+]	9 (±1,4)	ad – s
0,5 Zn^{2+} 10,0 Pb^{2+}	4 (±0,9) 6 (±1,9)	13 (±3,7)	ad
0,5 Hg^{2+} 0,05 CN^-	5 (±3,4) 13 (±3,3)	15 (±2,0)	n
0,5 Cu^{2+} 0,05 CN^-	3 (±3,2) 13 (±3,3)	2 (±2,4)	an
0,5 Zn^{2+} 0,05 CN^-	4 (±0,9) 13 (±3,3)	8 (±1,7)	an
0,1 Hg^{2+} 0,5 Cu^{2+} 0,5 Zn^{2+} 0,05 CN^-	0 3 (±3,2) 4 (±0,9) 13 (±3,3)	6 (±3,0)	an
1,0 Pb^{2+} 0,05 CN^-	3 (±0,8)[+] 13 (±3,3)	21 (±2,4)	s
10,0 Pb^{2+} 0,05 CN^-	6 (±1,9) 13 (±3,3)	22 (±2,8)	ad
0,5 Hg^{2+} 100,0 C_6H_5OH	5 (±3,4) 3 (±1,1)	5 (±1,4)	n
0,5 Cu^{2+} 100,0 C_6H_5OH	3 (±3,2) 3 (±1,1)	3 (±1,1)	n
1,0 Pb^{2+} 100,0 C_6H_5OH	3 (±0,8)[+] 3 (±1,1)	1 (±1,0)	n
0,5 Zn^{2+} 100,0 C_6H_5OH	4 (±0,9) 3 (±1,1)	14 (±3,4)	s
0,5 CN^- 100,0 C_6H_5OH	13 (±3,3) 3 (±1,1)	18 (±2,8)	ad
0,1 Hg^{2+} 0,5 Cu^{2+} 0,5 Zn^{2+} 0,05 CN^- 50,0 C_6H_5OH	0 3 (±3,2) 4 (±0,9) 13 (±3,3) 0	7 (±1,4)	an

*Concentrations only causing a slight change in oxygen uptake rate
[α]Effect after 5 min of exposure
[+]Stimulation rather than inhibition (Slabbert and Morgan, 1982)
Each result is an average of six tests. S.D. is given in brackets. S.D. for control results ±0,8
ad – additive; an – antagonistic; n – neutral; s – synergistic

$$\frac{100}{1} - \left[\frac{\text{Ratio between test and reference oxygen uptake rate}}{\text{Average ratio between control and reference oxygen uptake rate}} \times \frac{100}{1}\right]$$

Students' t-test was used to establish whether results were significantly different at the P = 0,05 level.

Discussion of results

A multitude of terms have been suggested to describe various combined toxicant effects (Sprague, 1970; Muska and Weber, 1977). In this study a combined effect is described as additive when the observed effect is equal to the sum of the individual toxicities, and neutral if due entirely to the toxicity of the dominant constituent. Partial additive interaction occurs when the observed effect is intermediate between neutral and additive toxicity. Interaction is antagonistic if the combined effect is less than the neutral effect, and synergistic if the effect is larger than the additive effect.

Tests were carried out on mixtures of high concentrations of chemicals (concentrations exhibiting a marked change in oxygen uptake rate) (Table 1), mixtures of low concentrations of chemicals (concentrations causing no or only a slight change in uptake rate) (Table 2) and mixtures of high and low concentrations (Table 3). Results show that the concentration of individual chemicals in a mixture plays an important role in the type of combined effect exhibited, which is in agreement with findings of others (Sprague, 1970; Dutka and Kwan, 1982; Herbes and Beauchamp, 1977; Stebbing and Santiago-Fandino, 1983). To mention but a few examples, mixtures of high concentrations of two or three of the metals Hg^{2+}, Cu^{2+} and Zn^{2+} (Table 1) showed additive or partial additive toxicity; and neutral toxicity occurred when using one metal at a high concentration and the other at a low concentration (Table 3) or using all metals at low concentrations (Table 2). A high concentration of Zn^{2+} mixed with a low concentration Pb^{2+} (Table 3) showed antagonistic interaction, using a low concentration of Zn^{2+} (Table 2) in combination with 1,0 mg/ℓ Pb^{2+} the effect was additive to synergistic, and in combination with 10,0 mg/ℓ Pb^{2+} the effect was only additive. Partial additive toxicity was obtained when mixing high concentrations of phenol and Zn^{2+} (Table 1), antagonism when using a low concentration of phenol and a high Zn^{2+} (Table 3) and synergism when both concentrations were low (Table 2).

Chemicals interact differently when used in combination with one another. Various metal combinations (Table 1) showed additive or partial additive toxicity. Mixtures of CN^- and metals showed antagonistic interaction (neutral toxicity in the case of Zn^{2+}) and phenol metal mixtures partial additive toxicity when using Hg^{2+} or Zn^{2+} (Table 1) and neutral toxicity when using Cu^{2+}. Mixing 1,0 mg/ℓ Pb^{2+} with 0,5 mg/ℓ Zn^{2+} (Table 2) produced an additive to synergistic effect, with 0,5 mg/ℓ Hg^{2+} a neutral effect, with 0,5 mg/ℓ Cu^{2+} an antagonistic effect, with 0,05 CN^- a synergistic effect and with 100 mg/ℓ phenol neutral toxicity. Additive interaction of the metals Cu^{2+} and Zn^{2+}, and of phenol and Zn^{2+}, was demonstrated by Lloyd (1961) and Herbert and van Dyke (1964), respectively, when studying the toxicity of these mixtures to fish. As in the present study, Dutka and Kwan (1982) observed additive toxicity when testing Hg^{2+} Zn^{2+}, Hg^{2+} Cu^{2+}, Zn^{2+} Pb^{2+} (when using 1,0 mg/ℓ Pb^{2+} and 5,0 mg/ℓ Zn^{2+} the *T. pyriformis* bioassay indicated antagonistic interaction) and Hg^{2+} Cu^{2+} Zn^{2+} mixtures using the Microtox toxicity test. The additive toxic effect of metals was also illustrated by the *Spirillum volutans* test in the same study. Similar to the authors' results (obtained with the 0,5 mg/ℓ Cu^{2+} 1,0 mg/ℓ Pb^{2+} mixture), Cu^{2+} Pb^{2+} mixtures showed antagonistic interaction when tested with two bacterial growth inhibition tests (Dutka and Kwan, 1982). Tests using 10,0 mg/ℓ Pb^{2+} in combination with 5,0 mg/ℓ Cu^{2+}, however, showed synergistic activity (Table 3). Results obtained with Hg^{2+} Pb^{2+} mixtures were also different from the findings of Dutka and Kwan (1982). A 1,0 or 0,5 mg/ℓ Hg^{2+} 1,0 mg/ℓ Pb^{2+} mixture showed neutral toxicity,

TABLE 3
THE EFFECT OF HIGH* AND LOW** CONCENTRATIONS OF SELECTED CHEMICALS ON THE OXYGEN UPTAKE RATE OF *T. PYRIFORMIS*[α]

Chemical (mg/ℓ)	% Inhibition Individual effect	% Inhibition Combined effect	Type of combination effect
0,5 Hg^{2+}	5 (±3,4)		
5,0 Zn^{2+}	38 (±4,6)	42 (±5,7)	n
5,0 Cu^{2+}	22 (±5,9)		
0,5 Zn^{2+}	4 (±0,9)	18 (±3,1)	n
1,0 Hg^{2+}	57 (±3,4)		
1,0 Pb^{2+}	3 (±0,8)[+]	60 (±4,8)	n
1,0 Hg^{2+}	57 (±3,4)		
10,0 Pb^{2+}	6 (±1,9)	74 (±3,4)	s
5,0 Cu^{2+}	22 (±5,9)		
10,0 Pb^{2+}	6 (±1,9)	54 (±5,4)	s
5,0 Zn^{2+}	38 (±4,6)		
1,0 Pb^{2+}	3 (±0,8)	25 (±2,8)	an
5,0 Cu^{2+}	22 (±5,9)		
0,05 CN^-	13 (±3,3)	18 (±2,4)	n
5,0 Zn^{2+}	38 (±4,6)		
0,05 CN^-	13 (±3,3)	25 (±4,8)	an
1,0 Pb^{2+}	3 (±0,8)[+]		
0,5 CN^-	71 (±1,4)	63 (±2,0)	an
10,0 Pb^{2+}	6 (±1,9)		
0,5 CN^-	71 (±1,4)	64 (±2,0)	an
0,5 Hg^{2+}	5 (±3,4)		
500,0 C_6H_5OH	30 (±4,4)	26 (±2,8)	n
1,0 Pb^{2+}	3 (±0,8)		
500,0 C_6H_5OH	30 (±4,4)	16 (±4,0)	an
10,0 Pb^{2+}	6 (±1,9)		
500,0 C_6H_5OH	30 (±4,4)	38 (±2,4)	ad
0,5 Cu^{2+}	3 (±3,2)		
500,0 C_6H_5OH	30 (±4,4)	22 (±2,4)	an
5,0 Cu^{2+}	22 (±5,9)		
100,0 C_6H_5OH	3 (±1,1)	30 (±1,8)	ad
5,0 Zn^{2+}	38 (±4,6)		
100,0 C_6H_5OH	3 (±1,1)	22 (±0,4)	an
0,05 CN^-	13 (±3,3)		
500,0 C_6H_5OH	30 (±4,4)	39 (±3,4)	p ad

*Concentrations exhibiting a marked change in oxygen uptake rate
**Concentrations only causing a slight change in oxygen uptake rate
[α]Effect after 5 min of exposure
[+]Stimulation rather than inhibition (Slabbert and Morgan, 1982)
Each result is an average of six tests. S.D. is given in brackets. S.D. for control results ±0,8
ad – additive; an – antagonistic; n – neutral; p ad – partially additive; s – synergistic

and a 1,0 mg/ℓ Hg^{2+} 10,0 mg/ℓ Pb^{2+} mixture synergism, as against the antagonistic interaction when using the Microtox and growth inhibition toxicity tests. In their studies on the combined effects of Hg^{2+}, Zn^{2+} and Pb^{2+} on the growth of populations of ciliates, Gray (1974) and Parker (1979) found that while many combinations had antagonistic effects, others had markedly synergistic effects. Studies with algae also showed that in certain cases metal combinations acted synergistically, in other cases the same metals acted antagonistically (Wong and Beaver, 1981; Rai *et al.*, 1981).

A combined toxic effect is not simply the result of exogenous interaction between chemicals, but can be interpreted in terms of the differing responses of organisms (Stebbing and Santiago-Fandino, 1983). When using different test parameters and test organisms to evaluate toxic interaction, differences in combined toxicity as observed previously may thus be expected. Changes in oxygen uptake rate of *T. pyriformis* reflect effects on metabolic functions as well as cell activity (Slabbert and Morgan, 1982). Furthermore, since oxygen uptake relates to the number of viable cells in a suspension, large changes in oxygen uptake rates (Tables 1, 3 and 4) not only express sub-lethal reductions but also changes in population levels (Slabbert and Morgan, 1982). Differences in combined effects when using various testing systems may also be due to the different exposure periods, seeing that this plays an important role in the type of combined toxicity observed. These effects may change with time (Stebbing and Santiago-Fandino, 1983). Dutka and Kwan (1982) examined changes in combined toxicity using the Microtox and *Spirillum* tests and various exposure periods. For comparative studies it is thus important to use a consistent contact time. During preliminary tests with the *T. pyriformis* bioassay, effects were determined at 1 min intervals over a 5 min exposure period according to the procedure previously described (Slabbert and Morgan, 1982). Changes in effects were observed in some instances. Responses stabilized after 5 min, however, and this contact time was thus selected as the most suitable for use in this study.

In some cases toxicants in a mixture may combine chemically. Doudoroff *et al.* (1966) have found that mixed solutions of cyanides and metals may result in addition of toxicity, or in very different effects if the metal and cyanide combine. The synergistic and antagonistic effects obtained in the present study with CN^- metal mixtures may accordingly indicate chemical interaction. Pb^{2+} easily formed precipitates when mixed with high concentrations of Cu^{2+}, Zn^{2+} and CN^-. This was the reason for not using this metal in combination with metal, CN^-, or phenol CN^- metal mixtures (Table 1). The highest Pb^{2+} concentration without precipitate formation in admixture with 5,0 mg/ℓ Zn^{2+} was 1,0 mg/ℓ. Both 1,0 and 10,0 mg/ℓ Pb^{2+} in a mixture with 0,5 mg/ℓ CN^- caused precipitation, but were nevertheless tested (Table 3). The 5,0 mg/ℓ Cu^{2+} 10,0 mg/ℓ Pb^{2+} precipitated within minutes after mixing. Results for this mixture were obtained by testing it immediately after preparation. By doing so a synergistic effect was observed. The mixture showed neutral toxicity if evaluated after precipitation. Because of the insolubility of the Pb^{2+} salts the highest concentration used in the present study was 10,0 mg/ℓ, causing only a slight change in oxygen uptake rate (6% inhibition).

The severe effects of mixtures of chemicals which may be

TABLE 4
THE EFFECT OF CHEMICALS AT CONCENTRATIONS SET AS GENERAL STANDARD FOR INDUSTRIAL EFFLUENTS, AND DILUTIONS THEREOF, ON THE OXYGEN UPTAKE RATE OF *T. PYRIFORMIS*[α]

Chemical	Concentration* (mg/ℓ)	Individual effect (% inhibition)	Concentration (mg/ℓ)	Individual effect (% inhibition)	Concentration (mg/ℓ)	Individual effect (% inhibition)	Concentration (mg/ℓ)	Individual effect (% inhibition)
C_6H_5OH	0,1	0	0,01	0	0,005	0	0,001	0
NH_3	10,0	0	1,0	0	0,5	0	0,1	0
BO_3^{3-}	1,0	2 (±1,2)	0,1	0	0,05	0	0,01	0
F^-	1,0	2 (±1,0)	0,1	0	0,05	0	0,01	0
As^{3+}	0,5	77 (±1,4)	0,05	9 (±2,2)	0,025	4 (±2,4)	0,005	0
Cr^{3+}	0,5	49 (±4,5)	0,05	6 (±3,0)	0,025	2 (±0,9)	0,005	0
Cu^{2+}	1,0	8 (±5,2)	0,1	0	0,05	0	0,01	0
Zn^{2+}	5,0	38 (±4,6)	0,5	4 (±0,9)	0,25	2 (±1,2)	0,05	0
Pb^{2+}	1,0	3 (±0,8)+	0,1	5 (±2,5)+	0,05	5 (±1,7)+	0,01	2 (±0,8)
CN^-	0,5	71 (±1,4)	0,05	13 (±3,3)	0,025	8 (±2,4)	0,005	0
Combined effect (% inhibition)								
All ten		100 (±0)		44 (±1,2)		15 (±2,0)		3 (±2,0)
First six		100 (±0)		—		16 (±5,1)		3 (±0,8)
Last four		34 (±1,6)		—		6 (±1,5)		2 (±1,2)

*General standard (South Africa, 1962)
[α]Effect after 5 min of exposure
+Stimulation rather than inhibition (Slabbert and Morgan, 1982)
Each result is an average of six tests. S.D. given in brackets. S.D. for control results ±0,7

present in industrial effluents, at concentrations set as standard for effluents discharged into receiving waters (South Africa, 1962), are shown in Table 4 (first column). Individually, some of the chemicals (As^{3+}, Cr^{3+}, Zn^{2+} and CN^-) had a seriously inhibiting effect, others had no (C_6H_5OH and NH_3) or only a slight effect (BO_3^{3-}, F^-, Cu^{2+} and Pb^{2+}). A mixture of all ten chemicals caused 100% inhibition, indicating an additive effect of toxicities. Mixing the first six chemicals in the table also produced 100% inhibition. Antagonistic interaction was observed when mixing the last four chemicals. The pH of the ten chemical mixture was 2,54, which could have contributed to toxicity according to tests by Slabbert *et al.* (1983). After a ten- and twentyfold dilution of the mixtures (second and third column) individual chemicals were present at concentrations causing only a slight or no change in oxygen uptake rate. The combined effect of all ten chemicals after a tenfold dilution was additive to synergistic, and after a twentyfold dilution additive. A twentyfold dilution of the six chemical mixture showed an additive to synergistic effect, and of the four chemical mixture a neutral effect. Hundredfold dilutions of the mixtures still produced slight changes in oxygen uptake rate (significant at the P = 0,05 level). Results illustrate that some of the chemical concentrations should at least be twentyfold lower to avoid not only large sub-lethal effects when in mixture, but also lethality (reduction in cell numbers), and a hundredfold lower to exclude sub-lethal toxicity. Tests with activated sludge effluent as solvent showed that the toxicity of the mixture was screened off by the presence of other dissolved constituents, especially when chemicals were present at low concentrations. The mixture prepared with the ten chemicals showed a toxicity (99% inhibition) similar to that obtained when using deionized water. A tenfold dilution showed a 29% inhibition, as compared with the 44% inhibition when using deionized water, the twentyfold dilution only a 3% inhibition (15% when using deionized water). No toxicity could be detected in the hundredfold dilution. When the activated sludge effluent mixture was diluted with deionized water the effect of the tenfold dilution was the same as when using deionized water as solvent (45% inhibition), the effect of the twentyfold dilution half of that (7% inhibition) and the hundredfold dilution showed a 3% increase in oxygen uptake rate.

Irrespective of the type of combined effect exhibited by mixtures of high concentrations of chemicals (antagonism, synergism, addition or neutral toxicity), after dilution of mixtures to obtain minimum responses individual toxicants were still detected at their minimum effective levels (Table 2), or below when a number of chemicals were tested as in the case of the CN^- metal and the phenol CN^- metal mixtures and the mixtures prepared with chemicals present in industrial effluents (Table 4). Fivefold dilutions of the CN^- metal and phenol CN^- metal mixtures given in Table 2 respectively produced a 2 and 3% inhibition in oxygen uptake rate. Low concentrations (individually no effect) thus add up to produce a toxic effect. As in the present study, Dutka and Kwan (1982) found that lower concentrations of some toxicants in combination have toxic effects much greater than higher individual concentrations. When testing mixtures of a number of chemicals at concentrations equivalent to their individual EC_{50} levels, using growth inhibition tests, definite synergistic effects were observed with all combinations tested. In combination EC_{50} concentrations equivalent to 1/60 to 1/3 000 of the original EC_{50} values were established.

Conclusions

This study showed that interactive effects of chemicals on aquatic organisms are complex. In some cases chemicals acted synergistically, in other additively, antagonistically or neutrally. In wastewater synergistic, additive, partially additive and neutral effects can generally be expected to outweigh antagonistic effects by far. This illustrates serious shortcomings in water quality standards based on limits for individual compounds. At the same time it shows that the ability of bioassays to detect combined effects is probably much more important than their failure to detect the very low limits recommended for some potentially harmful compounds in drinking-water.

The study once again affirmed that the *T. pyriformis* bioassay system is an excellent short-term environmental screening test which allows sub-lethal effects to be assessed easily and rapidly.

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