Methods for Assessing the Effects of Mixtures of Chemicals
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Estimating the Impact of Pulses of Mixtures of Chemicals upon Aquatic Organisms

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ABSTRACT

Most estimates of the hazard of chemicals to aquatic organisms are based on evidence that depends on information derived from exposure to a constant concentration of a single chemical of a single species. However, most industries discharge mixtures of chemicals that may vary enormously in both concentration and composition due to various production processes. These highly variable chemical mixtures enter aquatic ecosystems composed of an enormous array of species. Considerable uncertainty exists in extrapolating from steady-state concentrations of single chemicals to which a single species is exposed to a highly variable exposure of chemical mixtures upon an array of organisms that may vary widely in their sensitivity to the component chemicals and which also may act differently in the aggregate than when tested individually. This paper explores a possible test design and the difficulties involved in developing alternative strategies.

1 INTRODUCTION

An old tale relates the story of a policeman who found an inebriated gentleman crawling around a lamppost late one night. The policeman asked him what he was doing. The answer was, 'Looking for my keys.' The policeman asked, 'Where do you think you lost them?' The drunk replied, 'Up the street,' and the policeman asked, 'Why don't you search for them there?' The drunk replied, 'Because there isn't any light there.' Although we might chuckle at this folly, an uncharitable person might say that our present toxicological practices are not better than the situation just described.

The single species toxicity test is an illegitimate offspring of mammalian toxicity tests (Slooff, 1983). Slooff (1983) has a very interesting table in his publication that illustrates some difficulties encountered when following the practices of mammalian toxicity if the objective is to evaluate toxicant effects on biological systems

that are considerably more complex than a single species. The question of the adequacy of single species tests for estimating effects at higher levels of biological organization has been explored (Buikema *et al.*, 1982; Cairns, 1981; NRC, 1981). This question is examined elsewhere in this volume and, therefore, its consideration is inappropriate here. However, we wish to affirm our belief that merely solving the problems of estimating impacts of chemical mixtures will not resolve all the problems.

The issue of pulse or episodic releases of mixtures into the environment is beset with problems such as (1) effect of varying duration, frequency, and amplitude of pulses, (2) temporal variation in proportions of chemical concentrations in the mixture, (3) variation in proportions of floating, soluble, and suspended components, (4) probability that short-term toxicity tests, or the collection of grab samples of an effluent, may not be conducted during a pulse, (5) dissimilar partitioning and persistence of mixture components after they enter the natural ecosystem, (6) short- versus long-term effects on the ecosystems, (7) additive versus non-additive effects among mixture components and abiotic features of the ecosystem, and (8) interactions of varying mixtures and environments, especially in tidal or estuarine ecosystems. Problems 1–4 are dependent in part on the type of plant operation, waste treatment facility, and waste retention time prior to discharge into the environment. Problems 5–8 are concerned with the ecosystem response after discharge of mixtures.

2 TESTING STRATEGY

The type of question being asked will affect the study design. Probably the most severe effect of pulsing will be directly on the organisms near the point of discharge. These organisms will be exposed to the greatest variability in component concentrations and duration and amplitude of exposure. Mixing of the effluent and receiving system water downstream of the effluent and the interaction between the mixture and the abiotic and biotic components of the ecosystem will dampen the effects of pulsing. Short-term effects, e.g. lethality, will probably be manifested most nearest the point of discharge, while long-term effects, e.g. chronic or sublethal effects, will be more apparent downstream of the discharge area. Alabaster and Lloyd (1982) have demonstrated that fish lethal effects can be predicted from additive effects of chemicals; as such, this paper is more concerned with chronic or sublethal effects.

The problem of pulse or episodic variability in both concentration and quality of chemical mixtures can be operationally divided into two categories: (1) response to varying concentrations of chemicals and chemical mixtures, and (2) consequences of dissimilar partitioning and persistence of components of chemical mixtures when they enter a complex natural ecosystem. One might determine whether pulsing the concentration of a pure chemical has any marked

effect upon the response of the test organism. A number of alternative hypotheses might be tested in this regard: (1) responses of the organism as if the chemical were at an average of the concentrations to which it was exposed, (2) response of the organism to the highest concentration to which it was exposed, (3) response determined by the frequency, duration, and amplitude of the pulses, and (4) nature of responses determined by the type of physiological impairment that occurs. In some cases, subsequent events are of no consequence once the damaging threshold has been crossed; alternatively, recovery is possible if the exposure to the peak pulses is not of particularly great duration or concentration.

Complex chemical mixtures entering an environment will partition in different ways. Fugacity equations provide a suitable means of determining the types of environmental compartments that will be entered by various chemicals, and means exist for estimating their persistence in each of these compartments (Neely and Mackay, 1982). Of course, it is *sine qua non* that a particular chemical may enter more than one compartment and that biological, chemical, and photochemical transformations of various sorts may result in still other compartments being entered. Therefore, estimations of the effects of chemical mixtures on aquatic organisms once the processes of partitioning have occurred may not be particularly strongly affected by the pulses of the delivery system. The most likely factor to increase the importance of pulsing in making an estimate of hazard is the effect of the pulsing itself on transformation, persistence, and partitioning of chemicals.

On-line measurement of the effects of complex mixtures, i.e. effluents, has been studied quite extensively using various types of dilutor systems so these will not be discussed. Most of this type of research has been on acute and chronic effects on single species. Many workers have looked at the laboratory effects of mixtures with subsequent field verification (e.g. Buikema *et al.*, 1981a). A few authors have attempted to simulate mixtures in order to understand the short- and long-term effects on single species. These investigations have included the use of simulated refinery effluents (Buikema *et al.*, 1976, 1981b; Hall *et al.*, 1978; McGinniss, 1978) and power plant blowdown effluents (Garton, 1972; McGinniss, 1978). Honig and Buikema (1980) also evaluated effluent criteria using a simulated refinery effluent and artificial streams; the periphyton were tested for non-taxonomic parameters, which included dry and ash-free dry weight, chlorophyll *a*, ATP, and algal community composition. Studies with simulated mixtures, however, do not always take into account the variability of mixtures (e.g. Huber *et al.*, 1979), although some methods have been proposed to study this variability.

Functional toxicity tests are extremely rare, and most water quality standards are based on lethality, behaviour, growth, reproductive success, and other important characteristics of single species; few standards are based on community or ecosystem function. To our knowledge, no standard method (as defined by the American Society for Testing and Materials) exists for community or ecosystem function, although many tests have been proposed (Matthews *et al.*, 1982a). The

test proposed here will not resolve all these problems, but it does offer the opportunity to carry out an inexpensive multispecies test that uses colonization rate and a heterotrophy/autotrophy ratio as end-points. Most ecologists and community biologists recognize colonization as an important attribute of natural systems and that a balanced ecosystem is composed of auto- and heterotrophic components. We hasten to add that this test is not intended to replace the single species test but rather to add another dimension to the process of estimating the hazard of chemicals to other aquatic organisms.

3 TEST DESIGN

3.1 Community Function

Many functional tests that have been proposed to evaluate the impact of mixtures on communities and ecosystems have problems in design and data derivation, analysis, and interpretation (Matthews *et al.*, 1982b). The multispecies test proposed here utilizes a microbial community that inhabits the interstices and surfaces of sediments, algal and fungal mats, etc., of aquatic ecosystems. This community is composed of many trophic levels with representatives of protozoans, algae, fungi, bacteria, rotifers, copepods, etc. (Buikema and Cairns, unpublished; Cairns, 1971; Paul *et al.*, 1977).

When previously uncolonized substrates are placed into an aquatic habitat, the substrate is initially colonized by pioneer species, e.g. flagellates, and substrate inhabitants undergo succession until a mature stable community is achieved (Paul *et al.*, 1977; Yongue and Cairns, 1978). Colonization is an important process for the establishment and maintenance of diverse and stable communities. Further, the ability of a community to invade a previously uncolonized substrate or to recolonize previously perturbed areas is critical to the stability of the ecosystem. Any factor that affects the colonization and successional patterns or survival of critical species will affect community stability.

While the microbial community is composed of many types of organisms, only the colonization rate of protozoans is monitored. Rotifers exhibit similar colonization patterns, but their species numbers are about one-quarter those of protozoans (Buikema and Hoffman, 1986). Protozoans are particularly attractive for colonization studies because: (a) they exhibit high reproductive and dispersal rates (Cairns *et al.*, 1969; Yongue and Cairns, 1973); (b) their size allows entire communities to be transported to the laboratory and used in microcosms (e.g. Cairns *et al.*, 1980); (c) they are trophically diverse and may characterize the responses of an entire community more accurately than any singular taxonomic group; and (d) they generally exhibit the species equilibrium model of MacArthur and Wilson (Cairns *et al.*, 1969).

In colonization studies, the temporal pattern of species accrual is monitored (Figure 1). These data are then analysed to determine if they fit the non-linear,

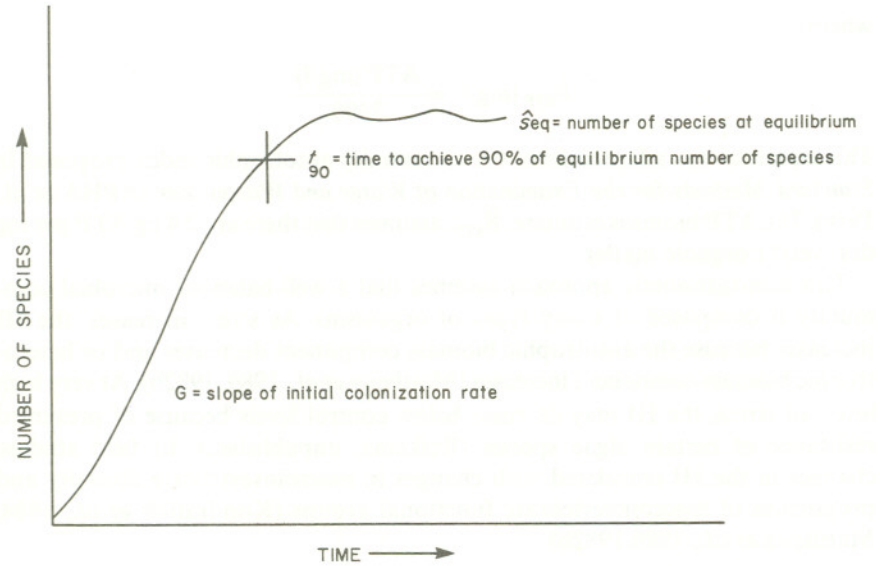

Figure 1 Hypothetical plot of protozoan species accrual on an artificial substrate over time

non-interactive colonization model of MacArthur and Wilson:

$$S_t = \hat{S}\text{eq}\,(1 - \text{e}^{-Gt})$$

where $\hat{S}$eq = number of species at equilibrium, G = constant for the initial rate of colonization, and t = time. A parameter, t_{90}, can also be used to estimate the time required to achieve 90% of the equilibrium number of species. Data that do not fit the non-linear model are assumed to be linear and do not come to equilibrium; they exhibit lack of fit (Cairns *et al.*, 1979; Draper and Smith, 1966).

In previous studies, increased toxicant stress resulted in a reduction in the initial rate of colonization (G) and an increase in the time to reach 90% of the equilibrium number of species (t_{90}), but not in the number of species at equilibrium. Similar patterns were exhibited for a pure compound (Cairns *et al.*, 1980), industrial effluents (Buikema and Cairns, unpublished), sewage treatment plant effluents (Cairns *et al.*, 1979), and stage of eutrophy (Plafkin *et al.*, 1980).

A second approach to evaluate the effect of complex mixtures on microbial communities utilizes a non-taxonomic approach. Biomass components are compared using chlorophyll *a* estimates of autotrophic biomass and ATP estimates of autotrophic and heterotrophic biomass. Biomass components are used to derive the heterotrophic index (HI):

$$\text{HI} = \frac{\hat{B}_{\text{ATP}}\,(\text{mg/l})}{\text{chlorophyll } a\ (\text{mg/l})}$$

where

$$\hat{B}_{ATP}\,(mg/l) = \frac{ATP\,(mg/l)}{2400}$$

This equation was adapted from the community autotrophic index proposed in *Standard Methods for the Examination of Water and Wastewater* (APHA *et al.*, 1976). The ATP biomass estimate, $\hat{B}_{ATP}$, assumes that there are 2.4 μg ATP per mg dry weight organic matter.

This non-taxonomic approach assumes that a well-balanced microbial community is composed of many types of organisms. As stress increases, the HI increases because the autotrophic biomass component decreases and/or heterotrophic biomass component increases (Matthews *et al.*, 1980, 1982b). At very high levels of stress, the HI may decrease below control levels because of presumed resistance of certain algae species (Buikema, unpublished). In field studies, changes in the HI correlated with changes in macroinvertebrate diversity and proportion of macroinvertebrate functional groups (Kondratieff *et al.*, 1984; Matthews *et al.*, 1980,1982b).

3.2 Colonization Studies

Basic strategies for protozoan colonization studies include placing polyurethane foam units (PFUs, $\sim 5\ cm^3$) *in situ* in a discharge gradient (Figure 2) at the end of

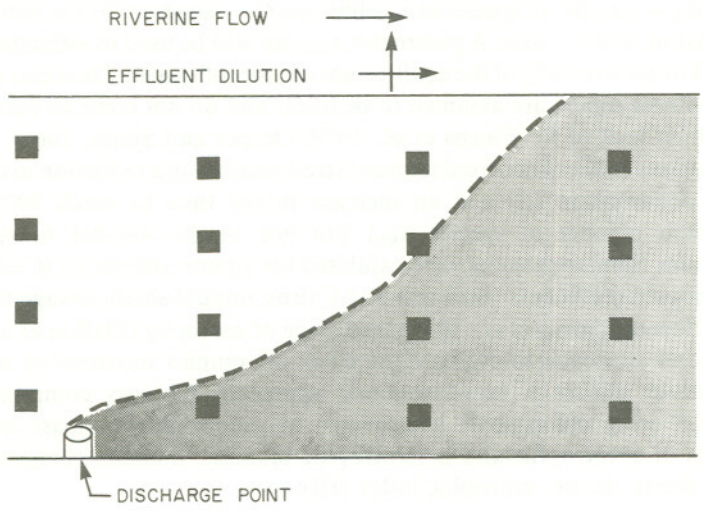

Figure 2 Possible placement of PFUs in a discharge plume to study the impact of mixture on colonization and heterotrophy

a continuous or intermittent flow toxicity test that uses serial or proportional dilutors, or in static systems containing various dilutions of a complex mixture. In field studies, the source of protozoan species for colonization is upstream of the point of discharge or from the surrounding environment at the *in situ* location.

In static or flow-through studies, the species pool is provided by PFUs previously colonized at reference sites (these colonized PFUs are known as epicentres) and then transported to the laboratory. The basic strategy for a static test involves placing uncolonized PFUs in a concentric ring about an epicentre (Figure 3) (e.g. Cairns *et al.*, 1980). The uncolonized PFUs are squeezed to fill the substrate with test solution; the epicentre is not squeezed. The environment is pasteurized water containing various dilutions of toxicant or mixture of toxicants; replicate aquaria are used for each test solution. The system may be covered to reduce invasion of airborne protozoans, and it is illuminated to prevent biased movement of flagellate protozoans.

In field and laboratory studies, the number of species invading the PFUs over time is determined by randomly selecting two to three sponges from each test chamber, squeezing each over a clean beaker, and examining the beaker contents for number of protozoan species observed for a fixed volume of extract or interval of time. The PFUs are returned to their original test containers. The colonization test requires only that the number of species or different 'types' be enumerated, and species of protozoan do not need to be identified.

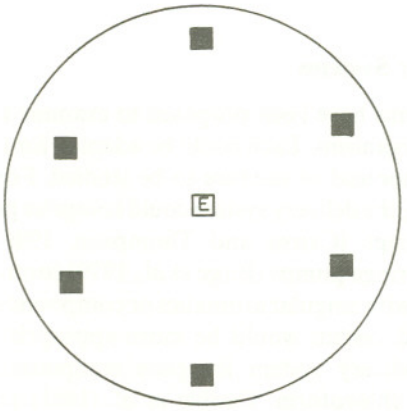

Figure 3 Placement of uncolonized substrates around an epicentre (E) to monitor mixture effects on colonization rate and heterotrophy

3.3 Heterotrophy/Autotrophy

When HI studies are conducted, PFUs are placed *in situ* for 7 days or for 15–21 days in laboratory studies. Again, the PFUs are squeezed when placed into the test medium. After defined periods of exposure, colonized PFUs are collected in Ziploc® bags and held on ice. The microbial community is extracted by squeezing the PFUs over clean glass beakers. The beaker contents are stirred, and aliquots for chlorophyll *a* and ATP analyses are collected and injected directly into appropriate solutions for extraction.

Adenosine triphosphate is extracted by injecting 1 ml of the microbial community into 9 ml of boiling Tris buffer [tris-(hydroxymethyl)aminomethane, 0.2 M, pH 7.75]. The direct injection of the microbial community eliminates the loss of ATP associated with filtration and simplifies the overall extraction process (Holm-Hansen and Booth, 1966; Jones and Simon, 1977; Tobin *et al.*, 1978). The samples are boiled for 15 min and frozen immediately at −20°C. Assays are completed using standard bioluminescence techniques with purified firefly enzyme on an ATP photometer (APHA *et al.*, 1976).

Chlorophyll *a* levels are measured by injecting 1 ml of the microbial community into 9 ml of 100% spectrograde acetone; this results in a 90% acetone extraction medium. The samples are extracted for 24 hours at 4°C in the dark (Parson, 1977). Chlorophyll determinations are made using a calibrated Turner Design fluorometer. The direct injection of the microbial community into acetone, without preliminary cell disruption through grinding or sonification, simplifies the extraction procedure (Holm-Hansen and Riemann, 1978).

Both chlorophyll *a* and ATP data are used to estimate biomass (see above), and the HI is computed. The HI is then compared to toxicant stress and protozoan colonization data.

3.4 Mixture Delivery Systems

Various delivery systems have been proposed to examine the effects of pulses of mixtures on aquatic organisms. Each could be adapted for research depending on the nature of the compound or mixture to be studied. For soluble components only, e.g. heavy metals, the delivery system could comprise programmed computer driven peristaltic pumps (Cairns and Thompson, 1980) or solenoid valves (Manley, 1980) and syringe pumps (Birge *et al.*, 1979) controlled by computers or timers. When dealing with singular aromatics or compounds containing a mixture of aromatics, a closed system would be more appropriate. Birge *et al.* (1979) designed a closed delivery system for pure compounds such as phenolics, phthalate esters, and chloroform. Dauble *et al.* (1981) proposed a system for studying the effects of water-soluble fractions of coal liquors, while Jenkins *et al.* (1977) proposed a similar type of system which could be used to evaluate the effects of water-soluble fractions of refined, crude, and waste oils.

Exclusive of proposed sediment bioassay test methods, no methods are known

for studying the effects of mixtures containing suspended solids. Continuous flow bioassays studying mixtures with a high suspended solids load generally require periodic manual cleaning. A potential test system with self-cleaning capabilities would be a modification of the self-cleaning single species test system used by Cairns and Gruber (1979).

3.5 Proposed Test System

The system proposed herein uses a continuous flow design characteristic of a stream or riverine ecosystem (Figure 4). This is a prototype of a system currently being designed in our laboratory. Dechlorinated water passes through a headbox containing PFUs precolonized in a variety of headwater streams in order to provide a large potential species pool for colonization. This water is pumped to a series of exposure systems containing pasteurized sediment (Figure 5). The system is perturbed with pure compounds or mixtures of toxicants of prescribed duration, frequency, and amplitude. The test chemicals are added to mixing boxes receiving dilution water with protozoans. After a pre-exposure period of 2–3 days, PFUs are added to the test chamber (Figure 5). Species accrual is monitored over time, and the HI is determined at the end of the 15- to 21-day exposure period.

The test chamber is designed to work with soluble, floating, and suspended components of a mixture (Figure 5). A baffle initially traps some of the floating component, and drains are available to remove excessive accumulation prior to PFU removal. Glass covers are provided over a minimal air space to reduce the loss of water-soluble fractions of organics. A magnetic stirrer ensures movement of water and suspended or flocculent particles over the substrates and sediment. The bottom sloping floor enhances migration of suspended particles downstream, and the small, reduced passage between the test container proper and the maintenance chamber also facilitates movement and removal of suspended particles. A cleanout port for suspended particles is also provided. The entire system is illuminated to facilitate colonization and prevent biased movement of chlorophyllous protozoans and algae. Sediments can be provided to simulate partitioning of mixture components.

4 APPLICATION OF TEST

The effect of mixture discharge on colonization rate and HI has not yet been studied with the flow-through system; however, the effect of mixture discharge has been studied on a local stream receiving storm water run-off and combined electroplating plant and sewage treatment plant effluents (Cairns and Buikema, 1983). Site A was upstream of the effluent, and sites B, C, and D were about 0.5, 3.0, and 6.0 km downstream of the discharge, respectively. During a 15-day period, the concentrations of metals were measured three times, and they varied within and among sites (Table 1). Each site had 20 PFUs, and protozoan species accrual was monitored at 0, 1, 3, 6, 9, 12, and 15 days. For each site, the data were analysed by a

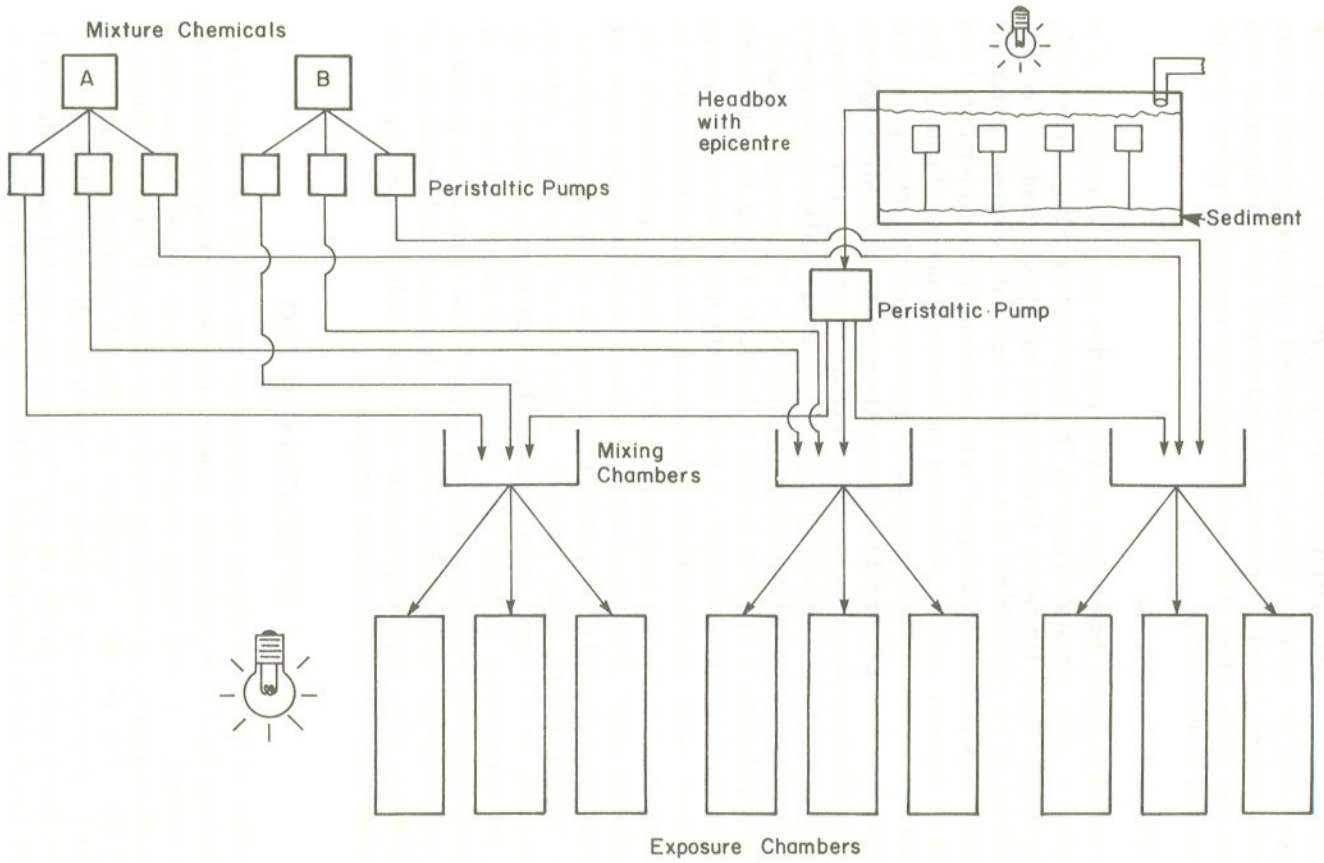

Figure 4 Delivery system to study the effects of mixtures and pulses on colonization and heterotrophy

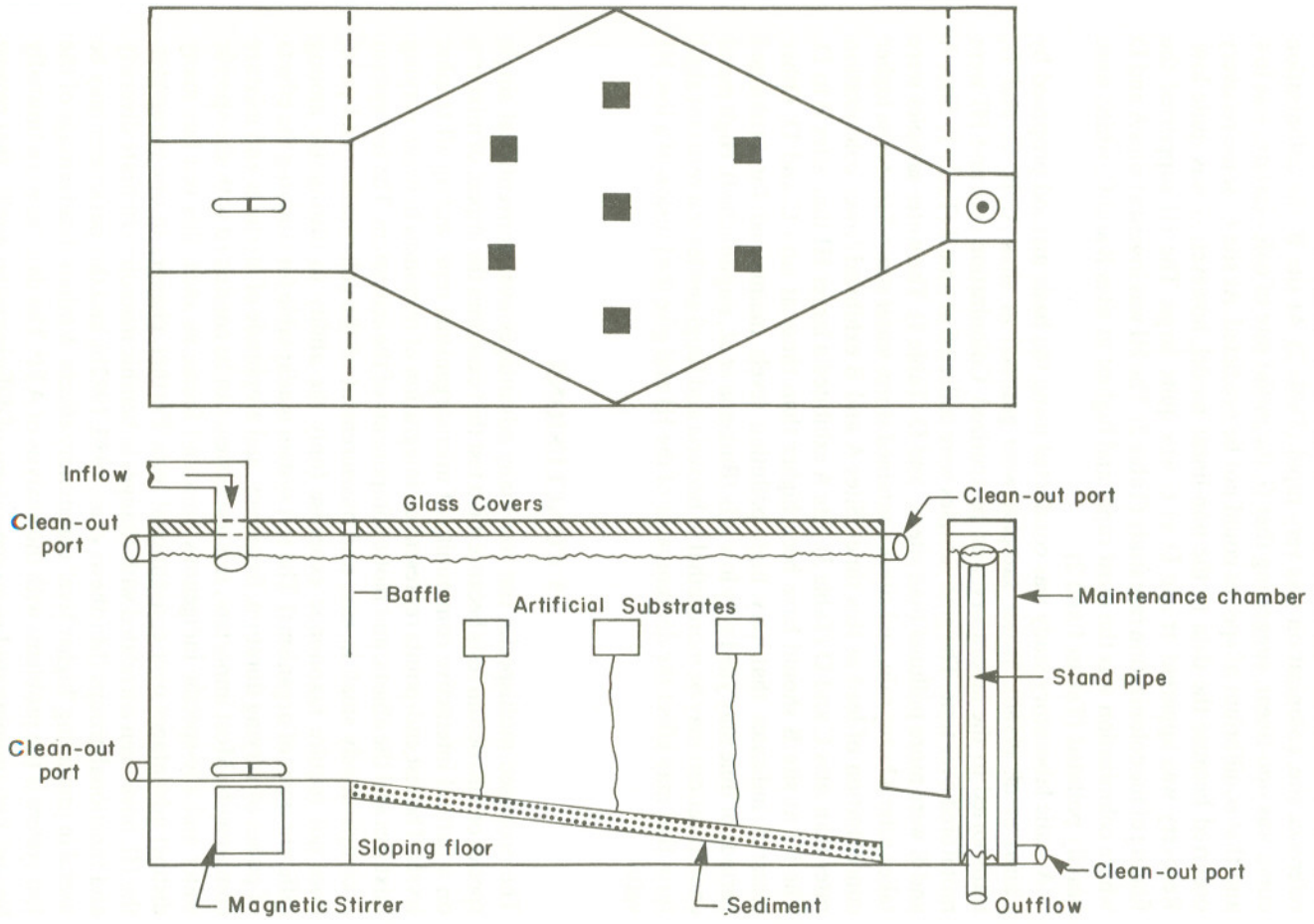

Figure 5 Diagram of exposure chamber used in laboratory studies on colonization and heterotrophy

non-linear model for colonization. The field data indicated that at site A, the reference site, colonization was very rapid (Table 2). At site B, the colonization curve was non-linear, suggesting that *G*, the initial rate of colonization, was low and that equilibrium of species could not be predicted. At site C, some recovery occurred because the data fit the non-linear model; however, *G* was quite low. Recovery was apparent at site D as *G* was quite large. The HI supported the finding of the colonization rate study (Table 2). The HI was lowest at sites A and D where colonization was the most rapid and highest at sites B and C, which were heavily polluted (Tables 1 and 2).

A static laboratory study was conducted using the basic method proposed by Cairns *et al.* (1980). Water samples were grabbed at sites A, B, C, and D, transported to the laboratory, and pasteurized. Colonization rate and HI were monitored over time. The grab samples were collected at time 2 (Table 1). Sites A and B were more polluted than sites C and D (Table 1). The water samples were taken after a heavy rain, and site A contained storm water run-off (note the higher concentration of lead at this time). Sites A and B exhibited lower colonization rates than sites C and D (Table 3). Site A exhibited a larger HI than sites B to D. The HI at site B should have been higher than those at sites C and D. Other research indicates that very high pollution levels maintained for prolonged periods of time may yield very low HIs (Buikema *et al.*, unpublished). High metal concentrations may be more lethal to bacteria and fungi and leave a resistant algal form that may affect the denominator of the HI and give the illusion of a low HI value.

5 CONCLUSIONS

The proposed multispecies test has many advantages over conventional single species or microcosm tests because it is a test that examines the impact of mixtures on a natural interactive community of microorganisms representing all trophic levels. The test end-points represent an integration of responses between varying mixtures and the abiotic and biotic components of the ecosystem. The test system is comparatively small in size, and measurement of the colonization end-point does not require taxonomic expertise (only the ability to distinguish among different types of organisms). The test system is adaptable for studying the effects of pulses of varying duration, frequency, and amplitude of mixtures and mixture components. Most important, this test system can be conducted with site-specific water and site-specific indigenous flora and fauna; as such, this test has many distinct advantages over conventional tests. Further, changes in one parameter, the HI, have been correlated with changes in benthic macroinvertebrate diversity and functional groups (Matthews *et al.*, 1980, 1982b), and this parameter may be useful in predicting 'higher level' community effects. A minor disadvantage of the test system is the problems with the analysis of ATP. The data base is admittedly limited, but on-going studies on ecosystems of differing water quality that receive

Table 1 Comparison of metal concentrations ($\mu g/l$) of an electroplating effluent at three different times during a two-week period

	Lead Sample date			Cadmium Sample date			Copper Sample date			Nickel Sample date			Zinc Sample date			Total Sample date		
Site	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
A	14	28	13	0.9	0.5	1.4	–[a]	0.4	–	19	18	25	<25	<25	–	<59	<72	39
B	40	82	90	4.1	0.7	1.9	48	93	276	33	21	10	57	<25	124	182	<222	502
C	50	25	38	1.1	1.7	1.9	16	24	96	16	6	28	14	12	59	97	69	223
D	–	9	2	–	1.1	0.9	–	0	4	–	7	30	–	1	25	–	28	<62

[a] Metal not detected or datum not available.

Table 2 Comparison of field microbial community responses to mixtures

	Parameter			
	Colonization rate			
Site	$\hat{S}eq$	G	t_{90} (days)	Heterotrophic index
A	26.2	607	<0.01	20
B	–[a]	–	–	275
C	23.8	0.06	38.3	710
D	26.0	24.9×10^{12}	<0.01	17

[a] Data are linear and do not fit non-linear model.

Table 3 Comparison of laboratory microbial community responses to mixtures

	Parameter			
	Colonization			
Site	$\hat{S}eq$	G	t_{90} (days)	Heterotrophic index
A	13.4	0.44	5.2	165
B	20.5	0.26	8.8	30
C	24.7	1.60	1.4	40
D	20.3	1.25	1.8	75

different types of effluents are being conducted to determine the utility of the proposed test.

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