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Microbiotests as tools for environmental monitoring

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INHOUDSOPGAVE

SAMENVATTING

In deze literatuurstudie is wordt nagegaan hoe microbiotestsystemen toepasbaar zijn als toxiciteitsindicatoren in milieumeetnetten. Onder microbiotestsystemen worden hier geminiaturiseerde aquatische ecotoxiciteitstoetsen begrepen die relatief snel, eenvoudig en goedkoop kunnen worden uitgevoerd. De uit de internationale literatuur verkregen testsystemen worden geëvalueerd en op basis van deze evaluatie wordt een minimum testbatterij van microbiotests voorgesteld. Deze testbatterij is zo ontworpen dat met grote zekerheid de meeste klassen van toxicanten kunnen worden gedetecteerd. De meeste behandelde testsystemen zijn gebaseerd op bacteriën die als respons op blootstelling aan toxische stoffen een vermindering van hun luminescentie laten zien, of een verandering vertonen in de aanmaak van bepaalde enzymen, hetgeen een kleurreactie teweeg brengt. Eveneens zijn onderzocht: een "microplate" algentoets, enkele geminiaturiseerde LC_{50} toetsen met evertebraten (Toxkits), enkele toetsen waarbij in evertebraten *in vivo* een enzymremming wordt gemeten, een teratogeniteitstoets met de larven van klauwpadden en een tweetal bacteriële genotoxiciteitstoetsen. Van elke test zijn de volgende eigenschappen onderzocht:

- het toetsgemak,
- de volledigheid van de documentatie,
- de kosten,
- de reproduceerbaarheid van de resultaten,
- de mate van standaardisatie van de test,
- de beïnvloeding van de resultaten door de toetscondities,
- de relatieve gevoeligheid voor milieumonsters en enkelvoudige zuivere stoffen.

Het onderzoek toont aan dat de meeste toetsen in de literatuur onvoldoende zijn gedocumenteerd. De geselecteerde testbatterij omvat het Microtox toetsysteem, de algen "microplate" toets, de Thamnotoxkit F met larven van een kreeftachtige en een bacteriële mutageniteitstoets (Mutatox of SOS-chromotest).

SUMMARY

In this study the usefulness of small-volume rapid aquatic toxicity tests (microbiotests) as environmental monitoring devices is reviewed. The individual tests are evaluated and a test battery is designed with the objective to be able to detect most classes of toxicants at low concentrations with a high reliability. Most of the discussed tests are bacterial luminometric or colorimetric assays for general toxicity determination. An algal microplate assay is also included as well as some miniaturized invertebrate LC_{50} tests (Toxkits). Additionally, invertebrate *in vivo* enzyme inhibition assays and a frog teratogenicity assay are evaluated. For the determination of genotoxicity, two bacterial assays are compared. For every test the following evaluation is made:

- Convenience (ease of operation, test duration, required manpower capacity and skill)
- Completeness of documentation
- Costs
- Reproducibility
- Level of standardization
- Influences of experimental conditions
- Sensitivity to environmental samples
- Sensitivity to a variety of single compounds

The results indicate that most tests are insufficiently documented. The designed battery includes the Microtox assay, the algal microplate assay, and the anostracan Thamnotoxkit F. Genotoxicity

may be measured reliably and cost-effectively by the Mutatox assay if the Microtox assay is also used.

1 INTRODUCTION

Over the past decades, public awareness of environmental pollution has grown. Pollution has become a major issue in politics, and legislation was introduced to protect water, air and soil quality. The monitoring and control of toxic substances has become an essential part of environmental policy.

The traditional approach to toxicant monitoring in water is chemical analysis, followed by comparing the observed concentrations to empirical no-effect levels for single compounds. This approach is inadequate and unpractical. Only a limited number of compounds can be evaluated this way due to the fact that full chemical analysis is either too costly or even impossible, but most of all due to the fact that the required (eco)toxicity data are relatively scarce. Furthermore, this approach neglects bioavailability, while synergism and antagonism among toxicants make mixture or combined toxicity unpredictable. These problems have been recognized by many authors (Roop and Hunsaker 1985, Felkner *et al.* 1988, Latif *et al.* 1993, Persoone and De Pauw 1991, Van der Wielen *et al.* 1993).

An alternative to chemical specific approach is ecotoxicological testing of environmental samples. The results are more realistic because bioassays integrate the effects of all bioavailable substances. Since individual species differ considerably in their sensitivity, batteries of tests must be used. Such a battery must be carefully composed. Several species and toxicity endpoints must be included (Dutka *et al.* 1991b) to obtain a maximal sensitivity spectrum. On the other hand, there are limits to manpower, equipment and financial resources. Therefore, tests must be selected that correlate as little as possible. Both the sensitivity spectrum and the redundancy increase with the number of tests. At a certain stage, an additional test will add more redundancy than sensitivity. The user's needs and resources determine the optimal number of tests.

Conventional aquatic bioassays, like the fish or cladoceran tests, are unpractical for routine environmental screening. Culturing and testing is expensive and laborious, as well as time and space consuming. Local differences in stock cultures increase the variation in the test results, and test organisms may not always be available. These drawbacks have been pointed out by several workers in this field (Persoone 1991, Persoone and De Pauw 1991, Blaise *et al.* 1986, 1988, Blaise 1991, Dutka *et al.* 1991b). This has led to the development of small-volume tests with small organisms, so-called microbiotests (Blaise 1991). They are designed as simple, cheap, standardized, quick and often culture-free tests. Some are commercially available as self-contained kits.

There are many microbiotests available, with different organisms and endpoints (Walker 1987, Blaise 1991). The aim of this review is to evaluate these tests, and to choose optimal combinations for environmental screening. The objective is, to be able to detect a wide variety of compounds at low concentrations with a high reliability and a minimum of effort and expenses.

2 METHODS AND CRITERIA

The information on the available aquatic microbiotests was gathered from public literature, dating back to 1980. A set of criteria was defined to select potentially useful tests:

- All tests must be acute whole organism tests which can be performed in a test volume of less than 100 ml. Small test volumes may reduce the costs of testing considerably. To evaluate low toxicity water samples, sometimes elaborate schemes have to be applied to concentrate the toxicants present. Testing the toxicological quality of sediment or soil with aquatic toxicity tests, requires time and money consuming extraction procedures. A reduction in test volume and thus in concentrate or extract volume requirements will save a lot of effort.

- The tests should preferably be available as a kit. A kit is a commercially available unit, that includes all the materials, reagents and media needed. Well-defined test organisms may be enclosed too. If tests are not available as a kit, their exposure time should be less than 24 h. Some of the selected tests do not meet this criterion, but are included as the sole representative of a large taxonomic group or a specific toxicity endpoint.
- The user has to possess only standard laboratory equipment, such as pipettes and an incubator, and sometimes a reading device such as a colorimeter. Tests should not require specifically trained personnel or extensive laboratory facilities. Tests involving radioactivity or histopathology are excluded.

The literature on the selected tests was studied, and additional information was requested from the original authors. A comparison was made of documentation, reproducibility, sensitivity, exposure time, standardization, technical simplicity and costs.

- Documentation is defined as the amount of information on test results, including information on reproducibility, and on the influence of test conditions.
- A test is considered sensitive for a group of compounds if its effect concentrations are lower than those of most other tests. Sensitivity to environmental samples is the ability to identify samples as toxic. For genotoxicity tests, sensitivity is defined as the ability to identify compounds as genotoxic. To assess the sensitivity spectra of the tests, two approaches were followed. First, results from environmental sample testing were compared. A test is considered sensitive to environmental samples if it has demonstrated toxicity at several occasions when other tests did not. Through this approach, differences can be demonstrated, but not explained. To gain more insight in the causes of differences among the tests, the second method involved the composition of a data base on the toxicity of pure compounds. The data for all tests except for the Microtox assay were collected from original publications. The data for the Microtox assay were looked up in data compilations, and additional Microtox data were collected from original publications. If more than one entry was found, the geometric mean was calculated.
  Genotoxicity data were treated in a similar way. Only data for the Mutatox assay were collected from the original literature. Matching data for the SOS chromotest and the Ames test were looked up in recent data compilations and some original articles. Data for the Ames test were used as a reference only.
- A test is considered standardized if detailed protocols are available. Preferably the test organisms should come from a common source. Media and materials should be well-defined. Tests in kit format are standardized by definition.
- Technical simplicity refers to the test volume and the complexity of organism handling and the preparation of test solutions. Kits with prefabricated media and not requiring culturing of test organisms are preferred.
- Prices in US $ are stated as specified by firms or authors. Only the expenditure on disposable materials and test organisms is taken into account, unless indicated otherwise. If the cost of individual measurements is specified in literature, the cost of a complete test is calculated as 18 individual measurements (five dilutions plus one control, in triplicate).

3 THE TESTS EVALUATED

Twenty-six tests are discussed in this review. All are aquatic tests or sediment tests; truly terrestrial tests were not found. Many are luminescent or colorimetric bacterial assays. Micro-algae tests are included in two versions. For invertebrates there are miniaturized lethality assays and rapid *in vivo* enzyme activity tests. The only teratogenicity test discussed is also the only vertebrate test. Two bacterial genotoxicity tests are reviewed. A concise overview of the tests is given in Table 1.

Table 1. Microbiotests: short description

NAME	SPECIES	MEASURE	ENDPOINT	METHOD
Bacteria:				
Microtox	*Photobacterium phosphoreum*	luminescence	respiration	tube
Microtox solid-phase	*Photobacterium phosphoreum*	luminescence	respiration	tube
Vibrio harveyi direct	*Vibrio harveyi*	luminescence	respiration	tube
Vibrio harveyi growth	*V. harveyi*	luminescence	population growth	tube
ATP-tox system	*Escherichia coli*	luminescence	ATP-content	tube
TOXI-chromotest kit	*E. coli*	coloration	enzyme synthesis	microplate
Sediment-chromotest kit	*E. coli*	coloration	enzyme synthesis	pad
MetPAD	*E. coli*	coloration	enzyme activity	pad
MetPLATE	*E. coli*	coloration	enzyme activity	microplate
ECHA biomonitor	*Bacillus sp.*	coloration	enzyme activity	dipstick
laser-microbe assay	*Bacillus subtilis*	light scattering	cell nr, size form	tube
Algae:				
Selenastrum microplate	*Selenastrum capricornutum*	cell count	population growth	microplate
Selenastrum ATP	*S. capricornutum*	luminescence	ATP-content	microplate
Invertebrates:				
Rotoxkit F	*Brachionus plicatilis*	mortality	general toxicity	microplate
Thamnotoxkit F	*Thamnocephalus platyurus*	mortality	general toxicity	microplate
Rotoxkit M	*Brachionus calyciflorus*	mortality	general toxicity	microplate
Artoxkit M	*Artemia salina*	mortality	general toxicity	microplate
IQ tests	*Daphnia magna*	fluorescence	enzyme activity	vial
	Ceriodaphnia dubia	fluorescence	enzyme activity	vial
	Mysidopsis bahia	fluorescence	enzyme activity	vial
	Artemia salina	fluorescence	enzyme activity	vial
Bacteria exoenzyme assay	local bacterial populations	fluorescence	exoenzyme activity	tube
Brachionus filtration test	*Brachionus calyciflorus*	fluorescence	substrate uptake	microplate
Teratogenicity (vertebrate):				
FETAX	*Xenopus laevis*	malformation	teratogenicity	petri dish
Genotoxicity tests (bacteria):				
MUTATOX	*Vibrio fischeri*	luminescence	genotoxicity	tube
SOS chromotest kit	*Escherichia coli*	coloration	genotoxicity	microplate/pad

3.1 GENERAL TOXICITY TESTS

Under this heading, microbiotests are summarized which are based on the detection of effects generally associated with impairment of biological membranes, enzyme inhibition or disruption of regulatory mechanisms. This type of toxicity leads to reduced metabolism and reproduction, and/or to the induction of mortality.

3.1.1 MICROTOX

The Microtox assay (Bulich 1979) is the most widely used microbiotest. It was commercialized in the early 80's (Bulich and Isenberg 1981, Microbics Inc., Carlsbad, CA, USA), and a huge literature collection has been built up (Microbics Inc. 1994). The test organism is *Photobacterium phosphoreum*, a luminescent marine bacterium. Luminescence in this species is a consequence of respiration, and its inhibition is a sign of toxic action. Because respiration is a process that takes place in every living cell, it is felt that Microtox results can, to an extent, be extrapolated to other species (Isenberg 1993).

The bacteria are stored in lyophilized form. When needed, they can be used after 5 minutes of rehydration. In a special apparatus the reduction in luminescence is measured after 5, 15 or 30 minutes of exposure. The results are usually expressed as EC_{50}, EC_{20}, EC_{10} or EC_{01} (the concentrations producing 50, 20, 10 or 1 percent of effect in terms of light reduction, respectively). Effects smaller than 50% increase the test's sensitivity but decrease its reliability (Microbics Inc.).

Sample color and turbidity are possible interferences (Kwan and Dutka 1992b). Freshwater samples must be osmotically adjusted with NaCl, which may alter the expression of toxicity.

As a major investment, the only equipment needed for this test is the Microtox apparatus with a cost of about $18,000. With a minor modification, the apparatus can also be used for execution of the Mutatox genotoxicity assay (see p. 7). After the purchase, the running costs per complete sample evaluation are about $5-15 (Aqua Survey Inc. 1992).

The Microtox can be automated: a self-supporting on-line continuous screening system is described (Levi *et al.* 1989). Also, a microplate version is presented (Blaise *et al.* 1994), allowing an increased sample throughput. In this application a microplate luminometer is used, automatically reading luminescence at previously set intervals. In the same paper it is suggested to prolong the exposure time to one hour in order to enhance sensitivity for some heavy metals in particular (copper and nickel toxicity increase tenfold relative to the 30 minutes value).

3.1.2 MICROTOX SOLID-PHASE TEST

Whole sediments can be tested according to the Microtox solid-phase protocol. The bacteria together with their medium are suspended with the sediment, and centrifuged after 15 minutes of exposure. The supernatant with the bacteria is pipetted into a cuvette, and the light emission is measured. Care must be taken with the selection of reference sediments. Internal standards, such as ^{14}C-labeled bacteria, must ensure that the bacteria are not spun down (Brouwer *et al.* 1990). The sensitivity range is rather narrow: sediments with EC_{50}'s over 2 volume percent are considered nontoxic (Kwan and Dutka 1992b). The cost of this test is $45 (Kwan and Dutka 1992b).

3.1.3 *Vibrio harveyi* ASSAYS

The *Vibrio harveyi* assays (Thomulka *et al.* 1993) are non-commercial bacterial luminescence tests. The test organism is a light-emitting marine bacterium, but contrary to P. phosphoreum, it can survive in oxygen-depleted environments. Under aerobic conditions it emits light as a consequence of respiration. A decrease in light emission is a sign of toxicity.

The test organisms are grown on agar, and prior to testing a watery culture must be prepared. Two test procedures have been described: a direct cytotoxicity test, and a population growth test. In the direct assay, an aerobic culture is incubated with the toxicant for 1 h before measurement. The nutrient medium is sufficient for catabolism, but cannot sustain growth. In the population growth assay an anaerobic culture is put into an aerobic toxicant solution, and after five hours luminescence is measured. For the population growth assay a rich medium is used (Thomulka *et al.* 1993).

The equipment required for these tests is an incubator and a luminometer. An anaerobic chamber is needed for the population growth assay (Thomulka *et al.* 1993). The actual cost of these tests is not calculated, but they are probably cheaper than the Microtox assay (Dr. K. W. Thomulka, pers. comm. 1994). Test organisms must be purchased once, but thereafter the cost of culturing is minimal. Cultures can be maintained at room temperature.

3.1.4 ATP-tox SYSTEM

The ATP-tox system (Xu and Dutka 1987) is a method that can in principle be performed with any bacteria or alga, and also with some yeasts. The authors use a freshwater version with *Escherichia coli* (strain K12 PQ37, from the SOS chromotest). The adenosine triphosphate (ATP) concentration, a

measure of physiological activity, is measured after toxicant exposure. This test was developed by researchers from the Canada Center for Inland Waters. No other users were found in literature.

Organisms from an agar stock culture are grown in a nutrient medium overnight. After exposure to the toxicant for five hours, an ATP-releasing agent is added along with luciferin and luciferase. In the presence of ATP as a source of energy, luciferase reduces luciferin, and light is emitted. Often the results are presented as the percentage of luminescence inhibition produced by undiluted samples, but an EC_{50} can also be determined. The ATP-tox system requires 1 ml of sample per tube. When applied to local microflora, ATP-tox is said to provide an "ecologically relevant" quality statement (Dr. B.J. Dutka, pers. comm., 1994).

A possible interference with the test system is a direct toxic inhibition of the luciferase enzyme. When luciferase is more inhibited than bacterial metabolism, false negatives may occur. To correct for this, a control is processed with an ATP standard (Xu and Dutka 1987). More controls must be taken for sample color (Kwan 1993). Measurements must always be taken in triplicate, because the results are highly variable (Dr. B.J. Dutka, pers. comm. 1994).

The equipment for this test is a luminometer and an incubator chamber. The complete testing of one sample costs about $30 (Dr. B. J. Dutka, pers. comm. 1994).

3.1.5 TOXI-CHROMOTEST

The Toxi-chromotest is a commercially available colorimetric bacterial assay kit (EBPI, Inc., Brampton, Ontario, Canada). The bacteria are mutant *Escherichia coli*, which are sold in a lyophilized state. They have permeable cell walls, which increases their sensitivity towards toxicants. The stress of rehydration also increases their sensitivity (Reinhartz *et al.* 1987). The toxicity indicator in the Toxi-chromotest is ß-galactosidase activity. In *E. coli*, ß-galactosidase is a non-constitutive enzyme which will artificially be induced during toxicant exposure. The activity is dependent on enzyme production and specific inhibition. Production is a measure of cell vigor, and specific inhibition is a direct toxic effect on the enzyme (Reinhartz *et al.* 1987).

During exposure, the enzyme is induced by isopropyl ß-D-thiogalactopyranoside. Rat liver microsomes (S9-mix) can also be added, to metabolize the toxicants. After one hour, lysis is induced, and the activity of ß-galactosidase is measured. Enzyme activity is determined by means of a chromogenic substrate. A yellow and a blue coloring substrate can be used. The usual endpoint is the EC_{20} or the minimal inhibitory concentration (Reinhartz *et al.* 1987).

The equipment for the Toxi-chromotest is an incubator and a colorimeter. When less accuracy is needed, the color intensity can be read by eye (Reinhartz *et al.* 1987). The cost of completely testing a single sample is about $50 (EBPI 1994).

3.1.6 SEDIMENT-CHROMOTEST

For the testing of sediments, an application of the Toxi-chromotest is developed (Kwan 1993). It was commercialized as the Sediment-chromotest (EBPI, Inc.). The bacteria are grown on a mini petri dish in direct contact with the sediment to be tested. After two hours of incubation a drop of the sediment-bacteria suspension is put on a filter containing the blue coloring substrate. If the filter does not change color, the sample is toxic. This makes the test insensitive: the endpoint is an EC_{100}. This test takes less than four hours, and actual labor is minimal (Kwan 1993). Sediments should be diluted at least 50 % (Kwan and Dutka 1992b).

The only equipment for this test is an incubator. The results are read by eye. Cost per sample is $46 (Kwan and Dutka 1992b).

3.1.7 MetPAD and MetPLATE

The MetPAD (Bitton *et al.* 1992a) and MetPLATE (Bitton *et al.* 1994) assays are commercially available colorimetric bacterial assay kits (Group 206 Technologies, Inc., Gainesville, FLA, USA). They contain freeze-dried mutant *Escherichia coli*. They are diagnostics for heavy metal contamination in aqueous solutions (Bitton *et al.* 1992a). The toxicity endpoint is specific inhibition of ß-galactosidase activity (Bitton *et al.* 1994). This enzyme is specifically inhibited by metal ions, but insensitive to many organic substances (Dutton *et al.* 1988). The difference with the Toxi-chromotest is, that the enzyme is already present in the freeze-dried bacteria, and is not induced during the test. Only specific enzyme inhibition, not overall cell vigor, is measured (Bitton *et al.* 1992a,b).

The methodology of MetPAD is very simple: bacteria are rehydrated, and added to the sample. After 90 minutes of incubation, a buffer is added. Drops of the suspension are dispensed on the assay pad. After another 30 minutes of incubation, purple coloration is assessed qualitatively or semiquantitatively, by comparison to a negative control (Bitton *et al.* 1992a,b).

To the MetPAD, a modification is made to obtain quantitative results. The result is a colorimetric microplate assay, known as MetPLATE. The methodology is similar to the Toxi-chromotest (Bitton *et al.* 1994). Sediment applications have not been described.

A possible interference in the two tests may be attributed to toxicity of non-metals, which may lead to false positives. According to the authors, the test is insensitive to non-metallic toxicants, however, they actually only demonstrated that the tests are insensitive to some organic compounds (Bitton *et al.* 1992b, 1994).

Both tests require an incubator, and MetPLATE also requires a colorimeter. A single measurement by either test costs $3 (Group 206 Technologies, Inc.), leading to a price tag of $54 for a full-scale test.

3.1.8 ECHA BIOMONITOR

The simplest method discussed here is the ECHA biomonitor, a commercially available test kit (ECHA Microbiology Ltd., Cardiff, UK). The test was designed as a test for industrial hygiene, to screen products and wastewater for biocide residues. The ECHA biomonitor is a dipstick impregnated with *Bacillus sp.* (Dutka and Gorrie 1988), supposedly *Bacillus cereus* (Blaise 1991), and a growth-indicating dye (tetrazolium). The pad turns red as a response to bacterial growth (Dutka and Gorrie 1989). Dehydrogenase inhibition is supposed to be the actual toxicity endpoint (Bitton *et al.* 1994).

For testing, the pad is dipped into the toxicant solution or squeezed with sediment for 10 seconds, and then placed in an individual incubator chamber for 24 hours. Bacterial growth inhibition is assessed by comparing the color of the pad to a color card, and indexed as nontoxic to extremely toxic. Hundreds of tests can be performed by a single person per day (Dutka and Gorrie 1989).

The only equipment for this test is an incubator. An individual dipstick costs $2 (ECHA Microbiology Ltd., 1994), so a full-scale test costs $36.

3.1.9 LASER-MICROBE ASSAY

The laser-microbe assay is an elegant method that measures changes in light scattering by the freshwater bacterium *Bacillus subtilis* under the influence of toxicants. The system can detect population growth effects, but can also detect changes as small as 5% in the size or shape of the bacteria. These changes in size and shape reflect failure of osmotic regulation (Felkner *et al.* 1989).

For testing, a bacterial suspension is illuminated in a laser photometer, and the scattered light is measured by a circle of fifteen detectors. Light intensity is plotted against scattering angle, resulting in a smooth wavy curve. The intensity of scattered light is a direct function of the number of particles, i.e. the number of bacteria, in suspension. The size of the bacteria and the size distribution of the population can be read from the curve displacement between small and large scattering angles (Felkner *et al.* 1989).

The bacteria are either wild type or engineered. The latter are made susceptible to specific toxic actions. A battery of isogeneic strains can be used to measure specific effects, such as several types of DNA-damage and cytotoxic effects (Felkner *et al.* 1988, 1989).

The procedure of this test is rather simple. Freeze-dried bacteria are rehydrated at 37 °C in a small volume (0.4 ml), and diluted to 10 ml. Then a preliminary reading is made to decide if the culture has the right bacterial density. If so, 0.1 ml of liquid sample, with or without S9-mix, is added. Measurements are made at 0 and 66 minutes. The results are processed by an on-line computer, and expressed as the relative increase of population doubling time. The assay is fast, taking only one hour, and processing up to 10 sample concentrations simultaneously (Felkner *et al.* 1988). The interpretation of test results may require training.

The laser-microbe assay needs specialized and expensive equipment for measuring and data processing. The cost of one test was estimated at $ 360 (Toussaint *et al.* 1992). This may be an overestimation, because the equipment is depreciated over only 100 assays. The running costs per sample are not specified.

Much remains unclear about this assay. The relationship between population growth and changes in size and shape were not elucidated, and no detailed protocols were found. No more articles were published and the authors did not respond to correspondence. It is conceivable that research has stopped.

3.1.10 *Selenastrum capricornutum* MICROPLATE ASSAY

The microplate assay with the unicellular freshwater alga *Selenastrum capricornutum* (Blaise *et al.* 1986) is a miniaturized and standardized version of a conventional method that is performed in flasks. The toxicity endpoint can be the cell concentration, or the ATP-content. The population growth version does not meet the criteria for selection, being too time consuming. It is included because this test is the only phytotoxicity assay available in micro-format.

Algae from exponentially growing cultures are incubated in microplates with continuous light. After 96 hours, the cell concentration is determined with a hemocytometer. The ATP-contents can be determined after four hours by luminescence, presumably after cell lysis and luciferin-luciferase addition. When an automated liquid handling system is used for the test, a daily throughput of 35 tests is within reach. The microplates must be kept in sealed bags, against volatilization and to ensure sterility (Blaise *et al.* 1986).

A promising refinement of the algal microplate method was described, using chlorophyll fluorescence as a measure of stress. This method is very rapid and convenient (Caux *et al.* 1992). Unfortunately, no experimental data were published yet. It will therefore not be discussed further.

Some technical difficulties go with this assay. Infections may have disqualifying effects (Van der Wielen *et al.* 1991). Heating or autoclaving of samples seems a good option, but it may alter the chemical composition of samples (Kwan 1989). Corrections must be made for nitrogen and phosphate contents, so chemical analysis is required after all (Blaise *et al.* 1986). To correct for sample color in the ATP-assay, proper controls must be taken (Kwan 1989).

An electronic particle counter or a luminometer, and an incubator are needed for this test. The cost of one test, including culturing and equipment is $700 (Toussaint *et al.* 1991). This is probably an overestimation, because equipment is depreciated over only 100 assays.

3.1.11 TOXKITS

The so-called Toxkits (Persoone 1991) are commercially available kits (Creasel Ltd., Deinze, Belgium) featuring polycellular animals. There are two rotifer kits: the Rotoxkit F (freshwater) and M (marine) with *Brachionus calyciflorus* (Snell *et al.* 1991a) and *B. plicatilis* (Snell *et al.* 1989b) respectively. There are two crustacean kits: the Thamnotoxkit F, with the freshwater *Thamnocephalus platyurus* (Centeno *et al.* 1994), and the Artoxkit M, containing the brine shrimp *Artemia salina* (Van Steertegem and Persoone 1993). Both species belong to the order of Anostracans, or fairy shrimps.

There used to be a Streptoxkit F, featuring the fairy shrimp *Streptocephalus proboscideus*. It was replaced by the Thamnotoxkit F, because the latter was more reproducible and more sensitive (Centeno *et al.* 1994). More kits are under development: a Phytotoxkit, a Ceriodaphtoxkit, a Protozoantoxkit (Blaise 1994), a *Daphnia magna* kit, and even a fish kit (Persoone, pers. comm. 1994).

The test protocols indicate that the tests require little time investment (1.5 man hours) and exercise. The test animals are hatched overnight from cysts. They are ready for use within 24 h time, except for the *Artemia* larvae that need 48 h to molt to instar 2-3 (Persoone *et al.* 1993b). The tests themselves are carried out in multiwell microplates. The endpoint is lethality, the results are expressed as 24 h LC_{50} (Persoone 1991).

The main drawback of the Toxkits is the strain of constantly looking through binoculars during scoring. Volatilization may be a significant factor because of the small volume. The kits should therefore be sealed properly. The only equipment is an incubator and a binocular microscope. Single complete tests cost $45 (Creasel Ltd., Deinze, Belgium).

3.1.12 IQ/Fluotox TESTS

A relatively new concept in toxicity testing is measuring substrate uptake and enzyme activity *in vivo* by means of fluorescent substrates. The IQ/Fluotox tests (Hayes *et al.* 1993, Janssen and Persoone 1993) are commercially available test kits (Aqua Survey, Inc., Flemington, NJ, USA). The principle of fluorescence testing can be applied to many species, both freshwater and marine. Currently tests with the freshwater *Daphnia magna*, *D. pulex*, *Ceriodaphnia dubia*, and the marine *Mysidopsis bahia* are commercially available (Aqua Survey Inc. 1994). Tests with *Hyalella azteca*, (Janssen *et al.* 1993), *Artemia salina* (Espiritu *et al.*, in press), *Streptocephalus proboscideus* (Hayes 1994) and the earthworms *Eisena* and *Lumbricus* for soil testing (Muller *et al.* 1994) are being developed.

The methodology of these tests is rather simple. For the test with *Daphnia magna*, ten animals are exposed for one hour to a concentration range of toxicant in 10 ml vials. Then a non-fluorescent substrate (methyl umbelliferyl galactose) is added, that is taken up and metabolized. The metabolites are fluorescent when exposed to ultraviolet light. Reduction of fluorescence is supposed to predict mortality on longer exposure. The effect is expressed as an EC_{50}, which is determined by counting the proportion of brightly fluorescent animals (Janssen and Persoone 1993, Hayes 1993). Official standardization is under way (ASTM 1994).

Some interferences with the results may occur: if the test organisms do not ingest the substrate, toxicity may be overestimated. That may occur if the test sample has nutritional value, leaving the animals well fed before the substrate is added. Turbidity may also interfere with the readings (ASTM 1994). Compounds may reduce substrate metabolism by acting as a competitive source of food (Giesy, in press), also resulting in an overestimation of toxicity. It is important to take proper controls against such effects. Furthermore, the visual assessment is somewhat arbitrary.

Although the test organisms can be obtained for free with the kit (Aqua Survey, Inc.), cultures may give better results. Shipment should be avoided for two reasons: animal handling may cause stress (ASTM proposal P235), and animals may not be readily available. However, when a culture is used, the advantage of this test over conventional lethality testing is smaller.

No equipment at all is needed for IQ testing. The UV lamp is enclosed in the Starter Pack. The cost of completely testing one sample is about $50 (Aqua Survey, Inc.).

3.1.13 OTHER INVERTEBRATE FLUORESCENCE TESTS

Some IQ-like tests are described in literature, that are still in an experimental phase. They were not yet used in test batteries, and so far have not been standardized.

A test using fluorescence to measure the activity of exoenzymes of mixed bacterial populations has been described. The activity of the exoenzymes phosphatase and protease is measured by means of a fluorescent substrate on cultures of local field populations, rendering an "ecologically relevant" test. Its validation status is low, results having been published on just four compounds. Fluctuations in initial

activity may greatly influence the results, but the EC_{50}'s are relatively stable (Tubbing and Admiraal 1993).

Recently a method is proposed to measure filtering activity, but not enzyme activity, in *Brachionus plicatilis* by means of a fluorescent substrate. Its No Observed Effect Concentrations (NOEC's), specified as the highest concentration tested where no effects can be observed, correlates well with 48 h reproductive toxicity, and may therefore possibly predict chronic toxicity. The time investment for this test is only 2.5 h, with a total test time of 35 minutes (Juchelka and Snell 1994).

An IQ-like *Brachionus calyciflorus* assay is described, measuring esterase or galactosidase activity after 1 h of exposure. Promising results of this test with ten compounds are published. Its NOEC's were below reproduction NOEC's for several compounds, indicating its sensitivity. The apparatus required is a microscope, a video camera, and a computer with software for image analysis (Burbank and Snell 1994).

3.2 TERATOGENICITY TESTING

Teratogenicity, or the ability to induce malformations in embryos, may have a considerable impact on natural populations (Bantle 1985). Teratogenicity is quantified as the Teratogenicity Index (TI), defined as the ratio of the LC_{50} and the malformation EC_{50}. A non-teratogenic compound will cause malformations at concentrations only slightly below the LC_{50}, resulting in a TI between 1 and 1.5 (Dawson and Bantle 1987), while teratogenic compounds are characterized by a higher TI.

3.2.1 THE FROG EMBRYO TERATOGENICITY ASSAY - *XENOPUS* (FETAX)

The Frog Embryo Teratogenicity Assay - *Xenopus* (FETAX) test is a non-commercial test with embryos of the South African clawed toad, *X. laevis*. An ASTM guideline (ASTM 1991) is available, along with a very detailed operational guide (Bantle *et al.* 1991). FETAX does not meet the criteria for selection, but it was included as the only teratogenicity tests. It is also the only vertebrate test.

For the FETAX test, a culture of adult frogs is needed. For standardization it is important to use genetically well-defined strains. When embryos are needed, the parents are injected with human gonadotropin in the dorsal lymph sac. This induces mating, and the egg laying begins after 9-12 h. The eggs are swirled gently to remove their jelly coat. Then the embryos are examined. Only normally developing blastulae are used. 20 embryos are put into a 10 ml petri dish containing a reconstituted freshwater, the so-called FETAX solution. After 96 h, when the embryos have become small tadpoles, the test is stopped. Every 24 h, the toxicant solution is renewed (ASTM 1991) and the effects are scored (Dawson and Bantle 1987).

An application of the FETAX assay is described, to model teratogenic effects in mammals. Before entering the mammalian embryo, the toxicants may be metabolized in the mother's body. To mimic this, the toxicant is metabolized *in vitro* by rat liver microsomes (S9-mix) (Fort *et al.* 1988).

FETAX has some drawbacks. The test takes rather long and extensive culturing is needed. One female may produce thousands of eggs at a time, but between matings both male and female animals must be rested 60 to 90 days (Bantle *et al.* 1991). Good training is important to obtain uniform results (Bantle *et al.* 1994b). From the point of view of animal welfare this test is questionable.

FETAX's technical requirements are modest: an incubator and a microscope is all the equipment needed. The cost has not been specified.

3.3 GENOTOXICITY TESTS

Genotoxicity may be quite important for both humans and ecosystems. Genotoxicants directly damage the DNA. In principle, an interaction of DNA with a single molecule of a genotoxic compound may be enough to cause a lesion and initiate a tumor or cause a mutation in the offspring. Therefore,

neither EC_{50}'s nor safe levels can be determined. Effects are mostly measured qualitatively (positive or negative), Sometimes a damaging potential per molar unit is indicated.

All genotoxicity microbiotests are based on the detection of mutagenicity in specific strains of bacteria. Many genotoxicants only damage DNA after metabolization in the liver. When these types of compounds are administered to bacteria, the result may be a "false" negative: the test is negative, but the compound will initiate tumors when administered to higher organisms. To imitate mammalian body conditions, the samples can be pre-exposed to rat liver microsome mix (S9-mix) in order to produce the genotoxic metabolites *in vitro*. Analogous, for modelling the metabolism of fish, the S9-mix can be prepared from fish liver homogenates.

The *Salmonella*/microsome plate incorporation test, better known as the Ames test, is used only as a reference. It is the standard test for mutagenicity. It does not meet our criteria: it takes 96 h and requires culturing. A serious problem is the necessity of sterile conditions, that is almost impossible when testing environmental samples.

The laser-microbe assay, that was described earlier, can also be used for genotoxicity testing (Felkner *et al.* 1989). No experimental results were found, so it will not be further discussed.

3.3.1 MUTATOX

Although reports about genotoxicity testing with luminescent bacteria date back as far as 1980 (Ulitzur *et al.* 1980, Ulitzur 1982, Ben-Itzhak *et al.* 1985), the commercially available Mutatox (Microbics, Inc., Carlsbad, CA, USA) was launched only in 1990. An engineered "dark" mutant *Vibrio fischeri* (strain M169) is used. *V. fischeri* is a luminescent marine bacterium that resembles *Photobacterium phosphoreum* (Kwan *et al.* 1990, Johnson 1993a). After toxicant exposure, light emission is measured. Any light emission must be caused by reverse mutations, and is therefore a measure of mutagenic activity.

The procedure of the Mutatox assay is described in great detail by Johnson (1993a). Lyophilized bacteria are rehydrated and exposed to the test substance. After 16-24 hours, light emission is measured in a modified Microtox photometer. The response of Mutatox can be read quantitatively in a dose-related manner. A confirmed result can be obtained within 24h (Sun and Stahr 1993).

The addition of rat liver S9-mix tends to raise the detection limits and decrease the uniformity of the results (Kwan and Dutka 1990). Mutatox's poor response to metabolic activation may be explained by suboptimal conditions for metabolization. The rat liver S9-mix is developed for the Ames test which is conducted at a temperature of 37°C, whereas Mutatox is conducted at 15°C. An alternative is presented, which uses channel catfish liver as an S9 source (Johnson 1993b). Similar problems are reported for the SOS chromotest, that also uses the "original" S9-mix (Quillardet and Hofnung 1993). Cytotoxicity can interfere with Mutatox's results. A sample that is not only genotoxic, but cytotoxic as well, may inhibit luminescence. A population growth test, based on cell density, can be performed as a control (Johnson 1993).

For Mutatox measurements an adapted Microtox reader is used. To the original apparatus a separate temperature-controlled incubation system is added. Modification of an existing Microtox reader will cost about $2000. Completely testing a single sample costs about $50 (Petromation BV, Rijswijk, The Netherlands).

3.3.2 SOS-CHROMOTEST

One of the most commonly used genotoxicity tests is the SOS chromotest (Quillardet *et al.* 1982), with *E. coli* strain K12 PQ37. The original test, performed in tubes, was miniaturized to a commercially available microplate kit (EPBI, Inc., Brampton, Ontario, Canada). The kit includes freeze-dried bacteria, and freeze-dried S9-mix (Fish *et al.* 1987). The original test, which is still in use, is reviewed extensively (Quillardet and Hofnung 1993, Mersch-Sundermann 1994). In the SOS chromotest, the activity of the SOS DNA repair system is measured. When the DNA is damaged, SOS enzymes proliferate. In the K12 strain of bacteria, ß-galactosidase production is coupled with the SOS response. Through the

deletion of a gen, spontaneous production of ß-galactosidase is made impossible. The activity of the enzyme after exposure is measured by adding a chromogenic substrate after cell lysis. Coloration is read and a dose-response curve can be established. Alkaline phosphatase activity is measured in a similar way, and the ratio of the two enzyme activities is the induction factor. The results are finally expressed as the SOS-inducing potential (SOSIP). The SOSIP is the increase in induction factor per nmol of toxicant (Quillardet and Hofnung 1985). The results are available within six hours (Fish *et al.* 1987).

Some care must be taken with the interpretation of the test results. Several complications through toxic action are possible. The alkaline phosphatase test is a control for general protein inhibition, and prevents false negatives (Quillardet and Hofnung 1985). False positives may occur if the enzymes are inhibited in different proportions. Therefore another control is necessary (Hoflack *et al.* 1993).

This control is rather complicated: positive and negative control substances are tested, and after two hours of incubation the sample is added. During the previous two hours the enzymes have had time to proliferate, so enzyme inhibition must be a direct toxic effect of the sample. If both enzymes are unaffected, or affected in the same proportion, the results are clear cut. If the enzymes are not affected in the same proportion, further experiments are required (Hoflack *et al.* 1993).

The kit's procedures have been described in great detail (Xu *et al.* 1989). The SOS chromotest kit contains two chromogens: the standardly used yellow one, and a blue one for yellow test substances (Fisch *et al.* 1987). The SOS chromotest can be used for direct sediment testing. The sediment suspension is successfully tested in the microplate without filtering or centrifuging (Dutka and Kwan 1991a).

The technical requirements of the SOS chromotest kit are a colorimeter and an incubator. The cost of a single complete test is $50 (EBPI, Inc.).

Automation is optional: an apparatus with software is devised to make dilutions, measure, and process the data (Janz *et al.* 1988, Nylund *et al.* 1992).

Another application is a spot test, which is labor-extensive. A drop of bacteria and toxicant suspension is put on a filter after the incubation time, and coloration indicates genotoxicity. The spot test is semi-quantitative, and fit for pre-screening (Quillardet and Hofnung 1985).

4 REPRODUCIBILITY AND THE INFLUENCE OF EXPERIMENTAL CONDITIONS

Before a test is taken in use, the reliability of its results should be assessed. Each test's indigenous variation and the influence of physico-chemical parameters should be studied. That is especially important when environmental samples are tested. The results should be comparable between different times and places, and artifacts must be recognized.

4.1 GENERAL TOXICITY TESTS

4.1.1 MICROTOX

For Microtox, both reproducibility and experimental conditions are studied extensively. Among laboratories, coefficients of variance (CV, the ratio of standard deviation and mean) of 16.5 to 113% are reported for a variety of compounds (Greene *et al.* 1985). The influence of test time, temperature, pH, salinity, hardness, and various buffers on cadmium, zinc, pentachlorophenol (PCP) and benzene toxicity is also studied. Especially temperature and buffer composition appear to be important. The presence of calcium appears to be antagonistic to both metallic and organic toxicity (Vasseur *et al.* 1986).

4.1.2 *Vibrio harveyi* ASSAYS

No data on the reproducibility or the influence of experimental conditions on the *V. harveyi* tests are published yet. The variation between duplicates are reported to be small: most standard errors are less than 10% of the mean (N=4) (Thomulka *et al.* 1993). An article on reproducibility is in the process of being published (Dr. K. W. Thomulka, pers. comm. 1994).

4.1.3 ATP-tox SYSTEM

No information of the ATP-tox system's reproducibility or influence of experimental conditions has been encountered in literature. From preliminary data, there appears to be a large variation between duplicates: CV's up to 70 % were found (Dr. B.J. Dutka, pers. comm. 1994).

4.1.4 TOXI-CHROMOTEST and SEDIMENT-CHROMOTEST

Data on reproducibility are lacking for the Toxi-chromotest. The only modulating factor studied is pH. It appears that at pH 5.5 the test is more sensitive than at pH 7.5, especially for hydrophobous pesticides (Reinhartz *et al.* 1987). About the Sediment-chromotest, no information was found on these topics.

4.1.5 MetPAD and MetPLATE

For the MetPAD and MetPLATE methods, no studies on reproducibility are found. For MetPLATE there appears to be little variation between duplicates. CV's are below 5% for cadmium, chromium (III), lead, mercury, nickel, and zinc. Only for copper the CV is reported 19% (Bitton *et al.* 1994). No studies of the influence of test conditions have been published for either test.

4.1.6 ECHA BIOMONITOR AND LASER-MICROBE ASSAY

No information is found about reproducibility and experimental conditions of the ECHA biomonitor and the laser-microbe assay.

4.1.7 *Selenastrum capricornutum* MICROPLATE ASSAY

The results of the *S. capricornutum* microplate assay indicate a good reproducibility, with intra-laboratory CV's of 11-41% (Blaise *et al.* 1986, Thellen *et al.* 1989). The EC_{50}'s for phenol and cadmium deviates 25 % from the mean among three laboratories (Thellen *et al.* 1989). From the same study, the influence of gas exchange and the addition of a chelator (EDTA) to the medium is reported. Gas exchange has little effect, but EDTA markedly decreases cadmium and phenol toxicity. A good concordance between results from the conventional flask assay and the microplate assay is demonstrated for phenol, some metals and herbicides (St. Laurent *et al.* 1992).

4.1.8 TOXKITS

The reproducibility of Toxkits has been studied extensively. International intercalibration exercises have been conducted on the Artoxkit M, the Rotoxkit F and the Rotoxkit M. The number of participating laboratories was 129, 120 and 170, respectively. For these studies, CV's of 47.1 % for the Artoxkit M, 48.5 % for the Rotoxkit F and 49.1 % for the Rotoxkit M are reported (Persoone *et al.* 1993). For the

Rotoxkit F and M, it appears that production, processing and storage conditions have no significant effect on the sensitivity for a variety of compounds (Snell *et al.* 1991a,b). The former Streptoxkit F has been studied in a similar way, with similar results (Centeno *et al.* 1993). No such results of the Thamnotoxkit F and the Artoxkit M are published.

4.1.9 IQ/Fluotox TESTS

A report on a *Daphnia* IQ ring test with 16 participating laboratories reveals a 31% interlaboratory CV in copper EC_{50}. Intra-laboratory CV's amount on average to 21.8%, while 13 laboratories exhibit less than 40% CV. Ten laboratories had no prior experience with the test method (Aqua Survey, Inc., 1993). In another study, the published average intra-laboratory CV's are 43% for *D. magna*, 26% for *C. dubia*, 23% for *S. proboscideus* and 27% for *A. salina* (Hayes 1994). Effects of experimental conditions have not yet been reported.

4.1.10 TERATOGENCITY: THE FROG EMBRYO TERATOGENICITY ASSAY - *XENOPUS* (FETAX)

A ring test, comprising seven participating laboratories, has been conducted with FETAX. As the first phase in this ring test, a training and protocol evaluation has been executed. Intra- and inter-laboratory CV's proved to range from 4 to 83% (Bantle *et al.* 1994a). Thereafter a double-blind test has been conducted, resulting in intra-laboratory CV's of 1-48 %. Most laboratories demonstrated CV's below 15 %. (Bantle *et al.* 1994b). The influence of the test medium has been investigated with four test compounds. It appears that a reconstituted freshwater medium, now known as FETAX solution, gives the best results (Dawson and Bantle 1987).

4.2 GENOTOXICITY TESTS

The evaluation of genotoxicity tests is somewhat different. Results are mostly qualitative. Experimental conditions are unlikely to change the response qualitatively, although they may modify its amplitude. This is reflected in the results: conflicting results are scarce.

4.2.1 MUTATOX

For Mutatox, no interlaboratory comparisons or studies on modifying factors are published. In the present survey, some compounds are found to be tested by more than one author, and no disagreements are detected. No publications are found on the influence of test conditions.

4.2.2 SOS-CHROMOTEST

In a review article, a survey of reproducibility has been made for the SOS-chromotest (Quillardet and Hofnung 1993). Out of 103 compounds, 21 prove to provide conflicting results between studies. For 19 of these, explanations could be found. A clear cut contradiction was only observed in two of these compounds. From a study on the influence of experimental conditions it appears that modifications in pH, cell density, and S9-mix composition influence the amplitude of the response (Mersch-Sundermann *et al.* 1993).

4.3 CONCLUSIONS

In conclusion, it appears that the Microtox assay, the *Selenastrum* microplate tests, the Toxkits, FETAX and the SOS chromotest are the only tests that have been thoroughly examined with respect to the variability and the reproducibility of the results. Results from all other tests must be treated cautiously.

5 SENSITIVITY

5.1 ENVIRONMENTAL SAMPLES

The objective of this study is an evaluation of microbiotests in environmental sample testing. It is good practice to test environmental samples in test batteries: the toxicity of a sample is evaluated with a variety of tests. The results from those studies that involved the tests discussed here, are summarized in table 2. The tests are ranked according to their sensitivity. Some tests have not (yet) been used for environmental testing. These are: the laser-microbe assay, the *V. harveyi* assays, and the IQ tests except *D. magna*. The SOS chromotest has been used extensively for environmental screening. The results are reviewed by Quillardet and Hofnung (1993). Only those results that can be compared to the results of other tests are discussed here.

Most of the results discussed are from a Canadian research group (Xu & Dutka 1987, Dutka & Kwan 1988, Dutka & Gorrie 1989, Kwan & Dutka 1990, Dutka *et al.* 1991 a,b, Dutka *et al.* 1993). A very similar study was performed by another Canadian group (Costan *et al.* 1994). The major part of these studies have been focused on determining the toxicity contained in rivers and lakes, except for one, which dealt with the toxic properties of industrial effluents. A great variety of samples have been tested: water and sediments were collected from urban, industrial and rural areas all over Canada. The samples were concentrated or extracted according to a number of methods. The samples or extracts were tested with various combinations of the Microtox assay, the ATP-tox system, the Toxi-chromotest, the Sediment-chromotest, the ECHA biomonitor, the *S. capricornutum* microplate tests, the SOS chromotest, and the Mutatox assay. Occasionally, conventional bioassays were used for comparison.

The results of the Canadian studies indicate that the ATP-tox system and the Microtox assay are the most sensitive microbiotests. The Toxi-chromotest was found to be less sensitive, but the results correlated well with the results of the ATP-tox system. In some cases the *S. capricornutum* microplate test, in either version, was reported the most sensitive. In other studies it was found to be much less sensitive than the other assays. This may be related to the species selectivity of metal toxicity. The ECHA biomonitor proved to be more sensitive than Microtox and *D. magna* in one study, but less sensitive in another. Often the *D. magna* acute lethality assay indicated a higher toxicity than any of the applied microbiotests. The SOS chromotest revealed genotoxicity more often than the Mutatox assay. When genotoxicity was detected, the Mutatox assay gave a stronger response.

The freshwater Toxkits were used for environmental screening in two studies, along with the Microtox assay and the conventional *D. magna* acute lethality test. The Thamnotoxkit F appeared to be the most sensitive freshwater Toxkit. It was more sensitive than *D. magna* in two studies (Latif *et al.* , Persoone *et al.* 1993 b). The Microtox assay was more sensitive than the Toxkits and the *D. magna* assay (Persoone *et al.* 1993b). The *D. magna* IQ test was compared with the *D. magna* acute lethality assay in two studies. The IQ test was most sensitive to soil elutriates (Paul *et al.* 1994), but the lethality assay was most sensitive to effluents of a chemical specialty plant (Fischer *et al.* 1994). To effluents of metal processing industries, these assays were equally sensitive (Fischer *et al.* 1994).

The FETAX assay was only compared with a fish teratogenicity assay. In one study FETAX appeared to be five times less sensitive than the fish test (Dawson *et al.* 1988), but in another study they were equally sensitive (Fort *et al.*, in press).

Table 2. Results of environmental sample testing, ranked according to sensitivity. 1 - most sensitive.

Origin	Ind. harbor	Industrial area lake		Various	Urban area river			Various sources all over Canada			Urban area river	
Type of sample	sediment	sediment	sediment	sediment	sediment	sediment	sediment	water	sediment	sediment	water	sediment
Extraction method	water	water	DMSO	water	water	DMSO	methanol	none	water	DCM	none	water
Microtox	2	-	1	3	1	1	1	2	1	1	1	-
ATP-tox	1	2	2								1	1
Toxi-chromotest					2	2	2	3	3	1	-	-
ECHA biomonitor				1								+*
Selenastrum ATP		-	1**								-	-
D. magna lethality		1		2				1	2	1	-	-
Mutatox					+	+	+	+	-	+	-	-
SOS chromotest		-	-								+	+
Reference	(1)	(2)	(2)	(3)	(4)	(4)	(4)	(5)	(5)	(5)	(6)	(6)

Origin	Tar sands area river					Tar sands area lake		Agricultural area river		Pulp mill effluents	
Type of sample	water	water	water	sediment	sediment	sediment	susp. sed.	water	water	fresh	biodegraded
Extraction method	DCM acid	DCM base	flash evap.	water	DMSO	DCM	DCM	flash evap.	other meth.	none	none
Microtox	-	-	-	-	-	4	1	3	3	2	2
ATP-tox	2	2	2	2	1	2	4	1	1		
Toxi-chromotest	3	3	3	-	2	3	3	2	2		
ECHA biomonitor	-	-	-	-	-	-	-				
Selenastrum ATP	-	-	3	-	-	-	-			1**	1**
D. magna lethality	1	1	1	1	3	1	2				
Mutatox	-	-	-	-	-	-	-	+	-		
SOS chromotest	-	-	-	-	+	-	-			+	+
Reference	(7)	(7)	(7)	(7)	(7)	(7)	(7)	(8)	(8)	(9)	(9)

Origin	Munc. waste	Industrial	Waste dumps	Pharm.	Soil (metals)	Chem. plant	Metal plant	Zinc mine	Soil (PAH)
Type of sample	effluent	effluent	leachates	effluents	elutriates	effluent	effluent	water	elutriate
Extraction method	none	none	water	none	water	none	none	none	water
Microtox			1		-				
Rotoxkit F	2	3	3	3					
Thamnotoxkit F	1	1	2						
Streptoxkit F			2	2					
Rotoxkit M									
Artoxkit M									
Daphnia IQ					1	2	1		
FETAX								2	1
D. magna lethality	2	2	4	1	2	1	1		
other conventional test				1				1	1
Reference	(10)	(10)	(11)	(12)	(13)	(14)	(14)	(15)	(16)

1 Xu & Dutka 1987
2 Dutka & Kwan 1988
3 Dutka & Gorrie 1989
4 Kwan & Dutka 1990
5 Kwan et al. 1990
6 Dutka et al. 1991 a
7 Dutka et al. 1991b
8 Dutka et al. 1993
9 Costan et al. 1993
10 Latif et al. 1993
11 Persoone et al. 1993
12 Van der Wielen et al. 1993
13 Paul et al. 1994
14 Fischer et al. 1994
15 Dawson et al. 1985
16 Fort et al. (in press)

* direct sediment contact
** 96 h growth assay

- : no toxicity found
+: toxicity in most samples

5.2 SINGLE COMPOUNDS

In this part of the study, data on general toxicity and teratogenicity are collected for 202 compounds. Genotoxicity data are discussed separately (see 5.2.12). The compounds were grouped according to their supposed mode of action. The inorganic substances are grouped as metals, miscellaneous non-metallic substances, osmotics (such as NaCl), and oxidizers. Organic compounds are classified according to their structure (Verhaar *et al.* 1992). Class 1, nonpolar narcotics, includes compounds a.o. aliphatic alcohols, ketones, and benzenes. Class 2, less inert narcotics, contains a.o. phenols and anilins. Class 3, aspecific reactive compounds, contains a.o. aldehydes, bromides, and various antibiotics. Class 4, specific toxicants, contains mainly drugs and pesticides such as pentachlorophenol, lindane and organophosphates. Many compounds could not be classified; they were grouped as "other". These were a.o. nitroamines, dinitroaromatics, organic acids, and detergents.

It appeared that toxicity data are scarce for most chemicals. No single compound has been tested with all available tests, and few tests have been used to test every class of toxicants. Only metals and "other" organics feature data for all tests. This makes comparison between tests difficult. Most conclusions are based on pairwise comparisons.

The data, which are group-wise summarized in Table 3, are given in Appendix 1.

Table 3: Differences in sensitivity to single compounds. See also in the text.

	Metals	Inorg-anics	Oxid-izers	Organic compounds: classes 1	2	3	4	"other"
Microtox EC50	-	+	-	+	++	+	+	+
V. harveyi direct EC50	+	0	0	+	0	+	0	+
V. harveyi growth EC50	-	0	0	+	-	+	+	+
ATP-tox system EC50	-			0	+		0	0
TOXI-chromotest EC20	-	+	0	-		+	0	0
MetPAD inhibition	-			-	-	0	-	-
MetPLATE EC50	+			-	-	0	0	0
ECHA biomonitor MIC	0					-	0	0
Laser-microbe EC50	++			++	++		++	++
Selenastrum N EC50	++		+	0	+		+	0
Selenastrum ATP EC50	+				-			0
Rotoxkit F LC50	+	+	0	+	-	-	+	+
Thamnotoxkit F LC50	+	+	+	-	++		+	+
Rotoxkit M LC50	-	-		0	-		+	+
Artoxkit M LC50	-	-	0	+	-	-	+	0
Daphnia IQ EC50	+	0	+	++	-	0	+	+
Ceriodaphnia IQ EC50	+		0		-	0		+
Artemia IQ EC50	-		0		-	0	+	-
Mysidopsis IQ EC50	-		0		-		0	0
Streptocephalus IQ EC50	+				-	0	+	+
FETAX EC50	-			+		0	+	0

++	very sensitive: more than an order of magnitude more sensitive than all other tests to at least one compound
+	sensitive: up to an order of magnitude more sensitive than all other tests to at least one compound
-	not sensitive
0	insufficient comparison

N.B. ratings may be based on very different ranges and numbers of observations. They should be treated as indications.

5.2.1 MICROTOX

The results of Microtox tests are very well documented and have extensively been reviewed before. Most of the data used here were collected from two data compilations (Kaiser and Palabrica 1991, Persoone *et al.* 1993b). Additional data were collected from original articles and smaller compilations (Chang *et al.* 1981, Dutka and Kwan 1981, Casseri *et al.* 1983, McFeters *et al.* 1983, Krebs, Greene *et al.* 1985, Tarkpea 1986, Ribo and Kaiser 1987, Xu and Dutka 1987, Walker 1987, Elnabarawy *et al.* 1988, Couture *et al.* 1989, Ankley *et al.* 1990, Calleja and Persoone 1992, 1993, Calleja *et al.* 1994, Terrell *et al.* 1994). For 118 out of 202 compounds in the data base, Microtox results are available. All classes of compounds are represented several times. The Microtox assay is not sensitive to metals, which is probably an effect of salinity. It is sensitive to some inorganics, and insensitive to oxidizers. Microtox appears to be sensitive to all groups of organic compounds, and especially to phenols. No single-compound results from the solid-phase test were found.

5.2.2 *Vibrio harveyi* ASSAYS

The *V. harveyi* assays were both conducted with more than 40 substances, from all groups (Thomulka *et al.* 1992, 1993). There are some differences between the two versions of the test, but for most compounds the toxicity data are within the same order of magnitude. The direct assay is sensitive to metals, which is exceptional for a marine assay. Their sensitivity to inorganics and oxidizers cannot be assessed. They are sensitive to class 1 and some class 4 organics, and some out of the rest group.

5.2.3 ATP-tox SYSTEM

Only 14 single compounds were tested with the ATP-tox system (Xu and Dutka 1987, Dr. B. J. Dutka, pers. comm. 1994). Inorganics, osmotics, oxidizers and class 3 organics are lacking. ATP-tox does not show great sensitivity to any single compound, except some phenols.

5.2.4 TOXI-CHROMOTEST and SEDIMENT-CHROMOTEST

For the Toxi-chromotest, data are available for 30 compounds (Reinhartz *et al.* 1987), from all major classes except class 2 organics. Many are uncommon compounds, which are not tested with the other test systems. The test is sensitive to some inorganics and class 3 organics. No single compounds results from the Sediment-chromotest were found.

5.2.5 MetPAD and MetPLATE

Seven metals have been tested with MetPAD and MetPLATE (Bitton *et al.* 1992a, 1994). MetPAD was not very sensitive, but MetPlate was among the most sensitive tests for some metals. For other compounds, there are few data available: some organics were tested to demonstrate that at high concentrations no effects are present. Their EC_{50}'s were not determined.

5.2.6 ECHA BIOMONITOR

Little information was found on the compound specific sensitivity of the ECHA biomonitor. There is a data sheet available from the manufacturer, with toxicity data for 64 commercial biocide formulations (ECHA Microbiology 1993). Only two of these can be compared to the sensitivity of the Microtox assay: Microtox appears to be more sensitive. The test is insensitive to mercury and dinitrophenol (Dr. B. J. Dutka, pers. comm. 1994).

5.2.7 LASER-MICROBE ASSAY

For eight compounds toxicity data have been published for the laser-microbe assay (Toussaint *et al.* 1992). Data on inorganic non-metals and class 3 organics lack completely. From class 1 and 2 organics only one compound is tested (Toussaint *et al.* 1992). The response to organotins is described in great detail, but not in toxicological terms (Felkner *et al.* 1989). The assay shows extreme sensitivity for those compounds tested. The difference with the next most sensitive assay amounts up to seven orders of magnitude. It is reported that cadmium, zinc and trinitrotoluene are detected at levels below 1 ng/l.

5.2.8 *Selenastrum capricornutum* MICROPLATE ASSAY

For the *S. capricornutum* microplate assay in the cell count version, toxicity data for 25 compounds are published (Blaise *et al.* 1986, Couture *et al.* 1989, Thellen *et al.* 1989, Blaise and Harwood 1991, Hickey *et al.* 1991, St. Laurent *et al.* 1992, Toussaint *et al.* 1992). Especially metal toxicity is well documented, as well as the response to some herbicides. Non-metal inorganics and class 3 organics are lacking. Only ten substances, mostly metals, were tested with the ATP version (Blaise *et al.* 1986). The population growth assay is very sensitive to metals and oxidizers. It is generally not very sensitive to organics, except herbicides. The ATP-assay's sensitivity is generally about one order of magnitude lower.

5.2.9 TOXKITS

For the Rotoxkit F results for 77 compounds are documented (Snell *et al.* 1991a, Calleja and Persoone 1992, 1993, Calleja *et al.* 1994, Toussaint *et al.* 1992, Persoone *et al.* 1993b, Porta and Ronco 1993), for the Thamnotoxkit F, 40 (Centeno *et al.* 1993, Persoone *et al.* 1993a,b), for the Artoxkit M, 59 (Calleja and Persoone 1992, 1993, Calleja *et al.* 1994, Espiritu *et al.* 1993, Persoone *et al.* 1993a,b, Toussaint *et al.* 1992), and for the Rotoxkit M, 19 (Snell and Persoone 1989, Snell *et al.* 1991b, Calleja and Persoone 1992, 1993). The Rotoxkit F and the Artoxkit M were tested with chemicals from all groups. For the Thamnotoxkit F, the only group completely lacking is class 3 organics. For the Rotoxkit M there is no information found on oxidizers, class 2 and class 3 organics. As expected, the freshwater toxkits are more sensitive to metals than the marine kits. All freshwater toxkits are selectively sensitive to some inorganic compounds. For the marine kits there is insufficient comparison available. The Thamnotoxkit F is sensitive to the oxidizer bichromate and to anilins. It is generally more sensitive than the Rotoxkit F. The Artoxkit M is sensitive to some narcotics. The Artoxkit M was the most sensitive test for several class 4 compounds, but the other toxkits were also sensitive to some class 4 organics. For the rest group, no generalizations can be made. The Thamnotoxkit F appears overall to be the most sensitive.

5.2.10 IQ/Fluotox TESTS

Among the IQ tests, the *Daphnia* IQ test is the only test that is reasonably well documented. This test is documented with the test results on 29 pure compounds (Janssen *et al.* 1993, Aqua survey 1993a, Janssen and Persoone 1993, Hayes *et al.* 1993, Terrell *et al.* 1994, Hayes/Janssen *et al.* 1994, Giesy 1994) from all groups except osmotics. For non-metal inorganics and class 3 organics, there is but one result. All other groups are represented several times. The test is sensitive to metals and bichromate, as well as class 1 and class 4 organics. It is especially sensitive to organophosphates. The sensitivity pattern for individual compounds of the other IQ tests is substantially less evaluated, with two or more groups of toxicants missing entirely (Hayes/Janssen *et al.* 1994). They appear to be slightly less sensitive than the *D. magna* IQ test.

5.2.11 TERATOGENICITY: THE FROG EMBRYO TERATOGENICITY ASSAY - *XENOPUS* (FETAX)

FETAX test data are collected for 35 compounds, but most of these are not tested in other assays (Courchesne and Bantle 1985, Dawson and Bantle 1987, Fort *et al.* 1988, Dawson *et al.* 1988, Fort *et al.* 1989, Fort and Bantle 1990a, Fort and Bantle 1990b, Plowman *et al.* 1991, Sunderman *et al.* 1991, Dawson 1991, Dawson and Wilke 1991a, Dawson and Wilke 1991b, Dresser *et al.* 1992, Bernardini *et al.* 1994, Bantle *et al.* 1994, Schuytema *et al.* 1994, Rayburn *et al.* 1994, Prescutti *et al.* 1994, Fort *et al.*, in press). The chemicals tested are either known as mammalian teratogens or as non-teratogens, and include metals, class 1, 3, 4, and "other" organics. There is little comparison with other tests, but what comparison there is indicates that FETAX is not very sensitive. The only exception seems to be its high sensitivity for ethanol.

5.2.12 GENOTOXICITY: MUTATOX AND THE SOS CHROMOTEST

For 57 compounds, results of the Mutatox test were found (Kwan and Dutka 1990, Johnson 1993, Sun and Stahr 1993, Legault *et al.* 1994). Among them were some oxidizers, many aromatics, drugs, pesticides, and mycotoxins. No attempt was made to classify them. Out of the 57, 40 were also tested with the SOS chromotest (Mamber *et al.* 1986, Quillardet and Hofnung 1993, Legault *et al.* 1994, Mersch-Sunderman 1994) and 53 with the Ames test (Kwan and Dutka 1990, Johnson 1992, Quillardet and Hofnung 1993, Legault 1994, Mersch-Sunderman 1994). No matching data were found for only two compounds. From the data presented in appendix 2, it appears that for the majority of compounds the three tests respond similarly. The 16 compounds on which the tests disagreed are shown in table 4. Mutatox appears to have the widest sensitivity spectrum of the three tests. For all but two of the ambiguous compounds, the Mutatox results are positive. The SOS chromotest has the smallest spectrum: for all but one of the conflicting test results, a negative is reported. The Ames test is intermediate, being 6 times positive, 9 times negative and once inconclusive.

The differences in response between the three tests may partly be explained by differences in processing and metabolizing technique, as was suggested for the differences between the SOS chromotest and the Ames test (Quillardet and Hofnung 1993). This hypothesis was confirmed in a study on the effect of modifications to the S9-mix on PAH genotoxicity in the SOS chromotest (Mersch-Sundermann *et al.* 1993). Another part of explanation may be attributed to differences in sensitivity to certain types of DNA damage.

Table 4. Conflicting results for genotoxicity.

	Mutatox			SOS chromotest			Ames test		
	-S9	+S9	unknown	-S9	+S9	unknown	-S9	+S9	unknown
9-aminoacridine			+			-/equi.			+
2-aminobiphenyl			+		+				-
azide			-			-			+
benzidine			+	-	-		+	+	
benzo-(e)-pyrene		+			-			+	
benzoin			+						equivocal
caffeine			+	-	-		-	-	
cyclophosphamide			-		-				+
eugenol			+						-
8-hydroxyquinoline			+			-			+
nalidixic acid			+	+					+/-
novobiocin			+	-	-				-
propyl gallate			+	-	-		-	-	
pyrene		+		-	-	equivocal		-	
safrole			+		-				-
2,4,6-trichlorophenol	+			-			-		

-S9: unactivated compound
+S9: activated by S9 mix
unknown: no information about the use of S9 mix

6 DISCUSSION

6.1 EVALUATION OF INDIVIDUAL TESTS

The results of this survey are summarized in table 5. Due to the heterogeneity of the original data, most scores are qualitative.

Table 5: Summary of results

	EXP. TIME (H)	TECH. SIMPL.	SENSITIVITY		DOCUMENTED ♣	STANDARDIZED	CULTURE	EQUIPMENT ♠	COSTS (US$)•
			SINGLE COMPOUNDS	ENVIRONM. SAMPLES					
Microtox$	≤½	++	+	++	++	+	no	-	5-15
Microtox s. ph.$	≤½	0		+	-	+	no	-	45
V. harveyi direct	1	-	+		+	-	yes	-	
V. harveyi growth	5	-	+		+	-	yes	-	
ATP-tox system	5	-	0	++	-	-	no	-	30
Toxi-chromotest$	1½	+	+	-	-	+	no	-	50
Sediment-chromotest$	1½	++		+	-	+	no	+	54
MetPAD$	1½	++	0	+	-	+	no	+	54
MetPLATE$	1½	+	+	+	-	+	no	-	54
ECHA biomonitor$	24	++	-	+	-	+	no	+	36
Laser-microbe	1	++	++		-	-	no	-	360♥
S. capricornutum growth	96	--	+	+	+	+	yes	-	700♥
S. capricornutum ATP	4	-	+		-	0	yes	-	
Rotoxkit F$	24	0	+	+	+	+	no	+	45
Thamnotoxkit F$	24	0	+	++	+	+	no	+	45
Rotoxkit M$	24	0	-		+	+	no	+	45
Artoxkit M$	24	0	-		+	+	no	+	45
Daphnia IQ$	1¼	+	+	+	0	+/-	yes	+	50
Other IQ tests$	1¼	+	0		-	+/-	yes	+	50
Brachionus filt.	½	+	+		-	-	no	-	
FETAX	96	--	⊖	0	++	+	yes	+	
Mutatox$	24	++	+	+	+	+	no	--	5-15
SOS chromotest$	6	+	0	+	++	+	no	+/-	50

$ commercial kit
♣ low requirements are scored as +
• the amount of available information about sensitivity and reproducibility
♠ cost of organisms, media and disposable materials, unless indicated otherwise
♥ including culturing and all equipment costs averaged over 100 tests. Thus calculated, Microtox costs $160.
⊖ mainly teratogens
++ very good + good 0 mediocre - bad

6.1.1 MICROTOX

The Microtox assay is among the most useful tests. Its speed and convenience are unsurpassed. It is very well documented, and completely standardized. Microtox is sensitive to many compounds, especially organics. The apparatus is expensive, but its versatility can be improved by installing the option for the Mutatox assay.

The Microtox solid-phase test may turn out to be a valuable tool for sediment testing, but it needs more study. Its sensitivity range is smaller than that of the Sediment-chromotest, because it only allows low sediment concentrations.

6.1.2 *Vibrio harveyi* ASSAYS

The *V. harveyi* assays are fast and sensitive to many compounds. They have not been used for environmental testing yet, so they cannot be evaluated at this point. They are potentially useful: especially the direct assay may be a cheaper alternative to Microtox testing. The tests are not quite convenient. Study on reproducibility and the influence of experimental conditions is needed.

6.1.3 ATP-tox SYSTEM

The ATP-tox system is very sensitive to field samples, but it is ill-documented. It is not quite convenient, and single readings are known to be inaccurate. It is promising because of its sensitivity, but more study is needed.

6.1.4 TOXI-CHROMOTEST and SEDIMENT-CHROMOTEST

The Toxi-chromotest is fast and convenient. Its technical requirements are moderate, if the results are read with the naked eye instead of a colorimeter. It seems to be not very sensitive. Additional studies are required to allow a proper evaluation.

The Sediment-chromotest is cheap, and extremely simple. Its sensitivity range is larger than that of the Microtox solid-phase test, because it allows higher sediment concentrations. It is ill-documented, so more study is required.

6.1.5 MetPAD and MetPLATE

The MetPAD assay is cheap, convenient, and reasonably fast. However, it is not very sensitive, not even to metals. Therefore it does not seem quite useful. MetPLATE is sensitive, but less convenient. More study is needed on reproducibility, experimental conditions, and the supposed insensitivity to non-metallic toxicants.

6.1.6 ECHA BIOMONITOR

The ECHA biomonitor is very simple, practical and cheap, but relatively slow. It can be used for testing undiluted sediments. Unfortunately, this test is ill-documented. There is insufficient information available to evaluate this test's sensitivity.

6.1.7 LASER-MICROBE ASSAY

The laser-microbe assay is able to detect toxicants in a short time at minute concentrations. It needs, and perhaps deserves more study. Research seems to have stopped in the development phase.

6.1.8 *Selenastrum capricornutum* MICROPLATE ASSAY

The *S. capricornutum* microplate assay is rather expensive, slow, and unpractical. It is well studied, and very sensitive to metals. The four-hour ATP-endpoint is not studied extensively, and the original authors stuck to the 96-hour cell density endpoint. The test requires culturing and sterile conditions, and a hemocytometer is needed to read the results. Corrections for eutrophicating substances must be made. Attempts should be made to improve or replace this test by a more convenient one.

6.1.9 TOXKITS

The Toxkits are practical and easy tests. They have a minor disadvantage in being somewhat slow. The kits are exceptionally well documented, and completely standardized. The freshwater kits, and especially the Thamnotoxkit F, are sensitive indicators of toxicity for both pure compounds and environmental samples. The saltwater kits are sensitive to fewer compounds, but equally practical.

6.1.10 IQ TESTS

Like the Toxkits, the IQ tests are very practical and easy. They are fast, but insufficiently documented. The *Daphnia* IQ test is sensitive to many compounds, but its sensitivity to environmental samples and the influence of test conditions are insufficiently documented. The test animals may cause complications: they must either be cultured or shipped.

6.1.11 THE FROG EMBRYO TERATOGENICITY ASSAY - *XENOPUS* (FETAX)

The FETAX is one of the best documented assays in this review. But, unless the user is specifically interested in teratogenicity, FETAX can not be recommended for routine environmental screening. It is insensitive, slow and complicated compared to the other tests. A culture must be maintained.

6.1.12 MUTATOX

The Mutatox test is a very sensitive and practical genotoxicity test. Its technical requirements are rather high. When a lab already possesses a Microtox photometer, the Mutatox application is a relatively small investment that may give great returns. The metabolizing technique should be optimized.

6.1.13 SOS CHROMOTEST

The SOS chromotest is a cheap, quick and easy genotoxicity assay. It is less sensitive than the Mutatox test, but it does not require expensive equipment. It is very well documented, and the methodology is optimized. It requires a colorimeter for quantitative assessment. A very practical and cheap spot test for prescreening is available. It can be applied to whole sediments.

6.2 THE SELECTION OF A TEST BATTERY

The aim of this study is to select a battery of tests. Because a battery includes several organisms and/or endpoints, it predicts ecological damage more reliably than single tests. This principle has been in use for years in the toxicological evaluation of compounds. The components of a battery must be chosen properly. The battery must be reliable, provide a maximum sensitivity spectrum, and results should be obtained with a minimum of effort and expenses. For reliability the backbone of the battery should be formed by some well-characterized tests. To ensure a large sensitivity spectrum, the test results should correlate as little as possible, for environmental samples and single compounds. A genotoxicity test must be included to predict genotoxic effects. For reasons of economy the number of tests should be minimal.

The tests that are proposed to form the backbone of the test battery, are the Microtox assay, the *Selenastrum* microplate assay, the Thamnotoxkit F, and the Mutatox test or the SOS chromotest.

The Microtox test is the best documented microbiotests, and it is sensitive to many organic compounds. In environmental testing it also appeared to be very sensitive. In this battery it performs the function of a general indicator for toxic interference with cell metabolism.

The *S. capricornutum* microplate assay is included to detect metals and phytotoxic substances. Due to the fact that the microplate technique only involves a reduction in volume, its sensitivity and compound specificity does not deviate from the much more laborious flask type of *Selenastrum* tests.

Theoretically, these two tests cover all categories of compounds. But in environmental testing, the 48 hr *D. magna* lethality assay often indicated toxicity in samples that were nontoxic to bacteria and algae (Dutka and Kwan 1988, Kwan *et al.* 1990, Dutka *et al.* 1991b). Microbial tests alone are therefore insufficient. Another test should be included, to detect toxicity to invertebrates. The Thamnotoxkit F should be preferred: it is the most sensitive Toxkit for single compounds, and it was shown to be at least as sensitive as *D. magna* to environmental samples (Persoone *et al.* 1993b, Latif *et al.*, in press).

For genotoxicity either Mutatox or the SOS chromotest can be used. When the Microtox test is used, the Mutatox assay should be preferred for practical reasons. Otherwise the SOS chromotest is better: it is better documented, has lower detection limits and the metabolizing technique is optimized.

The proposed backbone battery of tests is most likely sensitive to compounds from all classes, but can probably be improved. Other promising tests should be included, and after some time the test battery should be re-evaluated to see if the new tests add information. The original tests may be replaced by better new tests. At present, the best candidates seem the *V. harveyi* assay, the ATP-tox method, the MetPLATE procedure, and the *D. magna* IQ test.

For direct sediment testing only four tests are available: the Microtox solid-phase test, the Sediment-chromotest, the ECHA biomonitor, and the SOS chromotest. Their application for sediment testing is still in the stage of development and documentation is not yet sufficient to make an attempt for battery construction.

6.3 RESEARCH NEEDS

This survey of methods and results reveals that although there are many potentially useful tests, most have not yet been studied sufficiently. Before a test can be taken in general use, it must be well characterized. Its reproducibility and the influence of test conditions should be studied in great detail. This has been done for only eight of the tests; for all the others there are important lags of knowledge. Before further investigation has been carried out, their results should be treated cautiously.

Another problem is the dispersion of the test results on pure compounds. It would be desirable to establish international standards for ecotoxicological tests which are including standard test results for a fixed collection of compounds, selected from every toxicological group.

There is a need for reliable direct sediment microbiotests. Extraction procedures have some serious drawbacks. Extracts may contain solvents that may be toxic by themselves, or may interact with toxicity. Once diluted up to the maximum allowable concentration of solvents, the toxicant concentrations may also be too low for detection (Kwan and Dutka 1992). Finally, every extraction method will have a different compound selectivity and thus will give different results (Dutka and Kwan

1988, Dutka *et al.* 1991a,b, 1993, Kwan *et al.* 1990, Kwan and Dutka 1991b). Some evidence was presented for the insufficiency of sediment extraction methods. In four studies, whole sediments and their extracts were tested with the Microtox solid-phase test and the Sediment-chromotest. Whole sediments appeared to be more toxic than their extracts (Brouwer *et al.* 1990, Kwan *et al.* 1990, Kwan and Dutka 1992a, Kwan 1993). The extracts from sediments that were spiked with polychlorinated biphenyls appeared to be nontoxic, but the whole sediments caused dose-related toxicity in the Microtox solid-phase test (Brouwer *et al.* 1993). This inadequacy is held responsible for the differences found in the other studies too (Kwan 1993). On the other hand, the direct sediment tests are not too well characterized, and the possibility of artifacts cannot be excluded. Especially on these tests, more study is required.

Although there are many microbiotests available, it is not certain that they give a correct impression of the variation in sensitivity among all species possibly present in an ecosystem. There are no microassays for vascular plants, fungi, protozoans, coelenterates, sponges, molluscs, insects, or fish. It should be demonstrated that adding the results of tests with these groups of organisms yields no additional information; otherwise such tests should be developed.

Finally, the application of no single test or test battery is guaranteed to enable extrapolation to safe toxicant levels for all species or ecosystems at large. Especially for compounds with a specific mode of action, the effect on a given species may be unpredictable. Therefore the relationship between the response of the test battery and effects in the field should be investigated. Because toxicity is species-dependent, the effects on a single species may be unpredictable. Effects on the whole ecosystem, such as a loss of biodiversity, might be more directly related to measured toxic levels.

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APPENDIX 1

Appendix 1: Toxicity data as log(ug/l)

bold is lowest concentration *italic is >concentration*

compound	Microtox 5m	Microtox 15m	Microtox 30m	Vibrio direct	Vibrio growth	ATP-tox	Tox-chromotest	Tox-chromotest acid	Tox-chromotest +/-S9	MetPAD	MetPLATE	laser-microbe	ECHA biomonitor	Selenastrum N	Selenastrum ATP	Rotoxkit F	Thermotoxkit F	Rotoxkit M brackish	Rotoxkit M marine	Artoxkit M	Daphnia IQ	Ceriodaphnia IQ	Artemia IQ	Mysidopsis IQ	Streptocephalus IQ	Brachionus filtration 1h	FETAX LC50	FETAX EC50	FETAX LC50 +S9	FETAX EC50 + S9
count:	90	93	50	42	43	14	32	17	4	15	13	7	7	26	10	77	39	18	17	59	29	10	13	6	11	9	29	34	6	7
Metals																														
ALUMINIUM																3.48														
BARIUM		7.72														6.66				5.57										
BORIUM (III)														4.19																
CADMIUM	5.14	4.37	3.73	2.32	3.69	3.13		3.86		2.48	1.46	-4.28		1.74	2.82	3.60	2.30	4.67	4.75	5.29	2.57	3.41	4.66	2.36	2.71	2.40				
CHROMIUM (II)	5.81													1.79	1.91															
CHROMIUM (III)											3.84			2.02													3.06	2.60		
COBALT	5.20	4.20	3.45	1.56	2.60		4.44							1.57	1.72												5.77	3.16		
COPPER	3.37	2.84	2.40	2.20	3.78	4.08				2.70	2.34	-0.89		1.70	1.95	2.15	1.91	1.94	2.01	2.29	1.89	2.41	3.92	2.80	2.20	1.70	2.97	2.91		
IRON(II)	4.85	4.84														4.13	4.62		4.49	4.23										
LEAD	3.23	4.48	2.60	*1.67*	3.81					3.70	4.00					*3.60*	3.21	*3.60*												
LITHIUM		6.07														5.43				4.65										
MANGANE(II)		4.14														4.54	4.37			6.52										
MERCURY	1.67	2.07	1.46	0.30	2.08	2.16	1.95	1.64		2.70	1.58		3.80	1.29	2.77	2.97	1.60	1.79		3.04	1.31	1.66	3.16	2.03	1.20	2.00				
NICKEL		4.34	4.30			4.02				3.90	2.99			1.57	2.21	3.66	3.34	*4.30*												
SILVER	7.00		3.36				1.72										0.88	2.41	2.08		1.32									
STIBIUM			4.05														3.72													
THALLIUM(I)		6.26	5.96													3.94	2.51			3.58										
ZINC	4.44	3.50	2.83	2.04	3.54	3.37		4.04		2.70	2.04	-3.52		1.69	2.20	3.39	2.35	*3.68*		*4.53*	2.43	2.66	4.62	3.60	3.20		4.37	3.26	4.40	3.29
ZIRCONIUM	*3.63*													3.41																
Inorganics																														
AMMONIA	3.30															3.66	4.11	4.44	4.25	3.75										
AMMONIUM CHLORIDE	6.56																5.15			*6.00*										
ARSENIC TRIOXIDE	4.67	5.75		4.56	4.39											3.31				4.20										
ARSENITE	4.31	4.14	4.05				2.75	2.78																						
CARBON TETRACHLORIDE	3.75	5.26														6.33				6.56										
CYANIDE	4.00	3.63	3.51					1.85								4.31	2.11			4.80										
FERRICYANIDE Fe(III)(CN)6								4.11																						
FLUORIDE	7.08	7.08		*4.65*	*4.65*											5.87	3.31			5.26										
NITRATE	5.62			*4.79*	*4.79*											6.56	6.61													
NITRITE	5.62																3.53													
SELENITE								3.51																						
SELENIUM																1.20		4.23												
SULPHATE	7.23															6.57	6.59													
UREA	7.38																				6.61									
Osmotics																														
CALCIUM																5.89	4.63													
CHLORIDE	7.30															6.34	6.43													
MAGNESIUM																5.56	5.79													
POTASSIUM																5.94	5.61													
SODIUM	7.30															6.18	6.26													
Oxidizers																														
AZIDE	4.66	4.73	4.63	*4.61*	3.20		3.11	3.11						2.54																
BICHROMATE	4.96	4.43	4.15	3.66	3.11					4.72				2.39		4.23	2.24			4.33	2.72		4.52	4.40						
CHROMIUM (VI)																					2.92	3.11	4.62							
HYDROGEN PEROXIDE				3.26	3.45																									
HYPOCHLORITE				1.45	3.73																									

compound	Microtox 5m	Microtox 15m	Microtox 30m	Vibrio direct	Vibrio growth	ATP-tox	Tox-chromotest	Tox-chromotest acid	Tox-chromotest +S9	MetPAD	MetPLATE	laser-microbe	ECHA biomonitor	Selenastrum N	Selenastrum ATP	Rotoxkit F	Thamnotoxkit F	Rotoxkit M brackish	Rotoxkit M marine	Artoxkit M	Daphnia IQ	Ceriodaphnia IQ	Artemia IQ	Mysidopsis IQ	Streptocephalus IQ	Brachionus filtration test	FETAX LC50	FETAX EC50	FETAX LC50 +S9	FETAX EC50 + S9
Class 1 organics: apolar narcotics																														
ACETONE	7.20	7.23	7.24	7.48	7.10		7.40							4.86 noec		4.71														
BENZENE	4.56	5.16	5.01													*6.00*	*4.70*			*6.00*	3.91									
n-BUTANOL	6.53	6.57	6.57	6.06	6.05																									
CHLOROFORM	5.72	6.01	5.83							*6.51*						5.75	*6.00*			6.32										
DICHLOROMETHANE		4.98														6.02	*3.00*			4.86										
ETHANOL	7.55	7.43	7.54	*7.00*	6.79		7.48									7.42			7.16	7.42							6.06	5.91		
ETHYL ACETATE	6.72	6.76		6.81	6.44																									
ETHYL AMINE	7.49	7.49		5.64	5.66																									
ETHYLENE GLYCOL	5.81	5.81	5.79	*6.00*	7.25											6.16			6.16	6.16										
1-HEPTANOL		4.00																									5.24	4.63		
HEXANE																4.83		5.19												
METHANOL	7.35	7.61	7.51	*7.00*	7.28		7.06							4.86 noec		7.58			7.71	7.58										
METHYL AMINE	4.54			4.94	5.45																									
2-PROPANOL	7.55	7.36		*7.00*	6.51											7.34			7.49	7.34										
n-PROPANOL	7.25	7.10																			6.34									
NAPHTHOL	2.84	2.87	2.94																							2.90				
a-NAPHTHOL	3.36	3.80	3.45			4.63																								
1-OCTANOL	3.77	3.89	3.86									0.87				*5.00*				4.77										
PROPYLENE GLYCOL	7.58	7.46	7.43	8.00	6.98																									
TOLUENE	4.23	4.29	4.29													5.05	*4.70*				3.69									
1,1,1-TRICHLOROETHANE	5.03	4.28														6.61	*5.00*			5.21										
XYLENE		3.86														5.35				5.49						4.46				
Class 2 organics: less inert narcotics																														
ANILIN	4.61	4.64	4.03	6.22	5.57												2.93			3.09										
CHLORAL HYDRATE																					5.23									
o-CHLOROPHENOL						5.44																								
m-CRESOL	3.83	3.87	3.89		4.73																									
3,4-DICHLORO ANILIN	2.65	2.75	2.81													4.79	2.04				3.15	3.41	3.62		3.41					
2,4-DICHLOROPHENOL	3.67	3.70	3.74			4.00					*5.70*			5.01	4.73															
3,5-DICHLOROPHENOL	3.64	3.58	3.59			3.40																								
2,4-DIMETHYLPHENOL	2.65	2.94	2.69																	4.85						3.78				
NITROBENZENE	4.45	4.47	4.54											4.24																
p-NITROPHENOL	4.03	3.98	4.12			4.54				*5.04*																				
PHENOL	4.39	4.45	4.44	*5.00*	5.07	5.65				*6.46*	*3.70*	2.51		3.59	*5.00*	7.08	4.52	*2.60*		5.33	4.53	4.62	5.13	4.79	4.54	5.40				
2,4,6-TRICHLOROPHENOL	3.78	3.92	3.89			4.46					*7.00*																			
Class 3 organics: reactive compounds																														
2-ACETYLAMINOFLUORENE																											4.95	3.86		3.41
ACETYLHYDRAZINE																											7.09	4.60		
ALL-TRANS-RETINOIC ACID																												0.78		
6-AMINONICOTINAMIDE																											6.35	3.68		
ASCORBIC ACID				3.66	4.45																									
B-AMINOPROPIONITRILE																												1.76		
BENZO(a)PYRENE																											*4.00*	4.00	*4.00*	3.23
CHLORAMPHENICOL		6.23		*5.00*	2.20											6.31				6.32										
CYCLOPHOSPHAMIDE ACT																											6.14	5.58		
CYTOCHALASIN D																											2.66	2.06	2.91	2.74
CYTOSINE ARABINOSIDE																											6.73	5.86		
DIPHENYLHYDANTOIN		*7.00*														*7.00*				*7.00*							4.89	4.52	4.85	4.50
ETHIDIUM BROMIDE																											4.70	4.54		
FLUOROACETATE							5.19	3.36																						
5-FLUOROURACIL																											5.21	5.14		
FORMALDEHYDE	4.00	3.93	3.93	3.98	3.57		3.30			*5.97*	*6.00*		4.88								4.53	4.62	6.44		4.62					
HYDROXYUREA			5.80																								6.07	5.58		
ISONIAZID		7.04														6.49				6.48							6.95	5.96		
ISONICOTIN ACID																											6.50	6.10		
KATHON (886 MW)	2.02	1.75											2.49																	
KATHON CG													2.41																	
NALIDIXIC ACID				*5.00*	3.15																									
PATULIN	3.70	3.28					2.54	2.70	3.18																					
PHENYLHYDRAZINE	4.89	4.86	4.82				1.78																							
RIFAMPICIN																											*6.31*	*6.31*	6.14	5.70
SEMICARBAZIDE																												4/6.78		
TETRACYCLINE				*2.00*	1.98																									

compound	Microtox 5m	Microtox 15m	Microtox 30m	Vibrio direct	Vibrio growth	ATP-tox	Tox-chromotest	Tox-chromotest acid	Tox-chromotest +S9	MetPAD	MetPLATE	laser-microbe	ECHA biomonitor	Selenastrum N	Selenastrum ATP	Rotoxkit F	Thermotoxkit F	Rotoxkit M brackish	Rotoxkit M marine	Artoxkit M	Daphnia IQ	Ceriodaphnia IQ	Artemia IQ	Mysidopsis IQ	Streptocephalus IQ	Brachionus filtration test	FETAX LC50	FETAX EC50	FETAX LC50 +S9	FETAX EC50 + S9
Class 4 organics: specific toxicants																														
ACETYLSALICYLIC ACID		4.84														4.96			5.13	4.98										
ACTINOMYCIN D																											4.26	4.34		
AFLATOXIN B1	4.34	4.33					4.20	4.20	3.30																					
AMITRYPTILINE		4.33														3.76			3.76	3.76										
AMPHETAMINE SULPHATE																6.18				3.69										
AMPICILLIN				5.00	5.00																									
ATROPINE SULPHATE		6.73														7.19				5.51										
BENDIOCARB							4.78																							
BENTHIOCARB																	2.82													
BROMOXYNIL														3.46																
CAFFEINE	4.90	5.68														6.54				6.67							5.41	5.11		
CAPTAFOL	3.84																			3.29										
CARBOFURAN	4.60																				1.74									
CHLOROQUINE PHOSPHATE		5.85														6.31				3.64										
COTININE																											6.64	5.86		
CYANAZINE														1.35																
CYCLOHEXIMIDE																											2.20	2.08		
CYPERMETHRIN	3.73	3.82	3.93				4.00																							
2,4-D	4.01	4.44	3.92			4.10				2.60	5.30			1.38		5.34		5.78		5.17										
DEET	4.84																				4.69									
DELTAMETHRIN							3.30																							
DEXTROPROPOXYPHENE HCl		4.75														5.60				3.73										
DIAZEPAM	5.42	7.00														5.02			6.24	6.23										
DIBUTYLTIN				2.65	1.62																									
DIELDRIN							4.18																							
DIGOXIN	5.99	7.00														6.67			5.28	7.00										
DIPHENYLHYDANTHOIN																														
DIQUAT DIBROMIDE							4.04							0.69																
ENDOSULFAN							4.06																							
ENDRIN							5.48	3.48																						
GLYPHOSATE	3.89													3.89																
GROTAN	4.63	4.46																												
GROTAN BK													5.19																	
GROTAN FORTE													5.11																	
GROTAN HD2 + IMPURITIES	5.04																													
GROTAN TK2													4.70																	
HEXACHLOROPHENE		3.90														0.52				-3.90										
HEXAZINONE														1.44																
8-OH QUINOLINE	4.06	3.89	3.36	3.66	3.32																									
IMAZAMETHABENZ														4.96																
LINDANE	5.50	5.28					4.90	3.48		5.70						4.15				5.26	3.24		3.76		2.79					
METHANEARSENIC ACID							4.90																							
METHOMYL							5.48	2.95																						
METOLACHLOR														1.71																
Na-PCP	2.97	2.88														3.06	2.88	3.13	3.13		3.13		2.72	3.50	3.62					
NICOTINE		4.58														5.69				5.34							5.14	2.57	4.31	3.77
NOVOBIOCIN	4.46	4.41	4.33	5.00	1.90																									
ORPHENADRINE SULPHATE		5.89														4.97				4.05										
PARACETAMOL	6.05	6.02	6.00													6.24			7.57	6.12										
PARAQUAT	5.89	5.64														5.18	2.77			5.16										
PARAQUAT CHLORIDE	6.05																				4.22						3.90			
PHENOBARBITAL		6.48														7.00				6.71										
PICLORAM														4.36																
2-PROPYLPENTANOIC ACID																												4.65		
QUINIDINE SULPHATE		7.47														5.44				3.94										
STREPTOMYCIN				5.00	4.28																									
TEBUTHIURON	5.52													1.96																
THEOPHYLLINE		6.40														6.92				6.59										
THIORIDAZINE HCl		2.80														4.16				2.47										
TRIBUTYL TIN				2.49	1.32											1.28		2.48												
TRIPHENYL TIN HYDROXIDE							4.00																							
VERAPAMIL HCl		5.61														5.52				4.00										
WARFARIN		4.83														6.56				5.65										

compound	Microtox 5m	Microtox 15m	Microtox 30m	Vibrio direct	Vibrio growth	ATP-tox	Tox-chromotest	Tox-chromotest acid	Tox-chromotest +S9	MetPAD	MetPLATE	laser-microbe	ECHA biomonitor	Selenastrum N	Selenastrum ATP	Rotoxkit F	Thamnotoxkit F	Rotoxkit M brackish	Rotoxkit M marine	Artoxkit M	Daphnia IQ	Ceriodaphnia IQ	Artemia IQ	Mytelopsis IQ	Streptocephalus IQ	Brachionus 48hr rotifer test	FETAX LC50	FETAX EC50	FETAX LC50 +S9	FETAX EC50 + S9
org. P																														
AZINPHOSMETHYL							4.00	3.70																			3.79	3.26		
CHLORPYRIFOS	4.66															4.08										2.40				
COUMAPHOS							4.40																							
DIAZINON	5.56						5.60	4.18	4.70								4.18				1.82					4.30				
FENITROTHION																3.83	3.60													
MALATHION	5.03	5.21					4.78	4.48	6.00			0.20				7.96		4.77	4.66	5.81	1.34									
PARATHION	4.36																				2.50									
RONNEL							4.34																							
TRICHLOROFON																4.67														
Other organics																														
ACETIC ACID																					5.32	4.66	5.20		4.51					
p-AMINOPHENOL	2.96	2.91	2.89	0.95	4.83																									
BENZALKONIUM CHLORIDE							2.40														1.56									
BUTYRIC ACID																												5.58		
CETYLTRIMETHYLAMMON.CHLOR	3.99	2.89																												
DIESEL FUEL																1.80		2.54												
1-Cl-2,4-DINITROBENZENE	4.11	3.76	3.55													3.11		3.30												
2,4-DINITROPHENOL	4.00	3.96	4.03										4.69 (NOEC)																	
DINITROPHENOL	4.00	4.01		*5.00*	2.93																									
1,4-DIOXANE	5.79	5.82	5.87	7.20	6.81																									
DMSO	4.94	7.99	7.99				7.70																							
EDTA																					5.11									
HYDROTHOL										*4.72*																				
LINEAR ALKYL SULFNT 11/18																					5.07									
alpha-NAPHTHYL AMINE																				5.00										
OXALATE		*7.81*														6.56				4.62										
PENICILLAMINE																												6.22		
PENTANOIC ACID																												5.30		
POLYOXYETHYLENE ALCOLHOLS							3.40																							
SACCHARINE																											7.26	7.29		
Na-DODECYL SULFATE	3.54	3.23	3.08	*5.00*	4.89	4.00				*6.46*	*3.70*			3.98	4.75	3.15	4.16	3.74	3.65		5.14	4.66	4.84	4.34	4.51					
TETRAHYDROFURAN				6.60	5.67																									
TRICHLORO ACETIC ACID																	4.23				5.14									
2,4,6-TNT	4.30											-3.55				3.74				4.46										
TRITON DF																											4.50	4.20		
TRITON-X-100				*5.00*	4.89																									

APPENDIX 2

Appendix 2: genotoxicity data

	Mutatox			SOS chromotest			Ames test		
	-S9	+S9	unknown	-S9	+S9	unknown	-S9	+S9	unknown
ACETAMIDE			-						-
2-ACETAMIDOFLUORENE		+	+		+			+	+
ACRIDINE ORANGE			+		+				+
AFLATOXIN B1	-	+			+			+	
AFLATOXIN B2	-				+			+	
9-AMINOACRIDINE			+			-/eq.			+
2-AMINOANTHRACENE		+			+			+	
2-AMINOBIPHENYL			+		+				-
2-AMINOFLUORENE		+	+		+			+	+
2-AMINONAPHTHALENE		+						+	
2-ANTHRAMINE			+						+
AZIDE			-			-			+
1,2-BENZANTHRACENE			+		+				+
BENZIDINE			+	-	-		+	+	
7,12-DIMETHYL-B(a)A		+ wk.			-			+	
BENZO-(a)-PYRENE	-	+	+		+			+	
BENZO-(e)-PYRENE		+wk.			-			+	
BENZOIN			+						eq.
BICHROMATE	+			+			+		
CAFFEINE			+	-	-		-	-	
CAPROLACTAM			-						-
CAPTAN	+			+			+		
CHLORAMPHENICOL			-			-			-
CHROMIUM (III)	-			-			-		
CYCLOPHOSPHAMIDE			-		-				+
2,4-D	-			-			-		
1,2-DIBROMOETHANE	-			-			-		
DIOXANE			-						-
EPOXIDE AFLATOXIN B1	-								
ETHYL METHANESULFONATE	-		+	-		+	-		+
EUGENOL			+						-
FUMONISIN B1	+								
8-HYDROXYQUINOLINE			+			-			+
MANNITOL			-						-
MENTHOL			-						-
3-METHYLCHOLANTHRENE		+			+			+	
METHYL-IMIDAZO-QUINOLINE	-	+wk.							
MITOMYCIN C			+	+			+		
N-METHYL-N-NITRSOGUANIDNE	+			+			+		
NALIDIXIC ACID			+	+					+/-
4-NITROQUINOLINE--OXIDE	+		+	+			+		+
NOVOBIOCIN			+	-	-				-
NTG			+						+
OCHRATOXIN A	-			-	-		-	-	
ORANGE G			-						-
PHENOL			+						-
PROFLAVINE	+		+		+			+	
PROPYL GALLATE			+	-	-		-	-	
PYRENE		+		-	-	eq.		-	
QUINACRINE			+						+
RESERPINE			-		-	-			-
SAFROLE			+	-	-				-
SALICYLIC ACID			-						-
1,1,1-TRICHLOROETHANE	-			-			-		
2,4,6-TRICHLOROPHENOL	+			-			-		
TRYPTOPHAN PYROLISATE P2	-	+			+			+	
TRYPTOPHAN PYROLYSATE P1	-	+							

+ genotoxic
- not genotoxic

+ wk. weakly genotoxic
eq. equivocal