THE USE OF ALGAE IN BIOASSAYS TO DETECT THE PRESENCE OF TOXIC COMPOUNDS IN NATURAL WATERS

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J A VAN DER HEEVER AND J U GROBBELAAR

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EXECUTIVE SUMMARY

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Supplementary to WRC Report No. 393/1/95 which is the full report to the Water Research Commission on the Project: "The use of algae in bioassays to detect the presence of toxic compounds in natural waters."

EXECUTIVE SUMMARY

Although effluent standards are at present based only on chemical and physical parameters, the results obtained from the monitoring of effluents and receiving waters may indicate the desirability of including biological parameters. Bioassays are necessary in water pollution evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota. Both biological and chemical evidence is needed to assess risk to ecosystems effectively. The ability to detect a compound does not ensure that biological effects can be predicted and the failure to detect a released chemical does not preclude its effects. Different kinds of aquatic organisms are also not equally susceptible to the same toxic substances.

The aims of this study were, therefore:
1. To use physiological indicators of algae to determine the presence of toxic substances.
2. To compare the response of algae to commonly used zooplankton toxicity tests.
3. To possibly develop a bioassay method which is cost effective and reliable.
4. To make recommendations as to which toxicity bioassay methods are the best suited to detect specific compounds.

In order to achieve this, the following, five different basic procedures were evaluated with six known toxic substances namely; copper, cadmium, mercury, atrazine (an organochloride), gusathion (an organophosphate) and phenol, as well as tertiary treated municipal sewage effluent. *Selenastrum capricornutum*, *Chlorella vulgaris* and *Daphnia pulex* were used as test organisms. The conventional EC50 value was used to indicate toxicity.

1. Algal Growth Potential (AGP) - This method has been in use for many years to indicate biostimulation and toxicity. Although it is time consuming it was used as a reference for comparative purposes.

2. ^{14}C-uptake rates by algae - This method is based on the techniques used to measure phytoplankton primary productivity. From the ^{14}C-uptake rates different algae and conditions could be compared and the presence of inhibiting factors and it should be possible to show the presence, or not, over a relatively short period.

3. O_2-evolution rates - Photosynthetic rates could also be measured in terms of oxygen liberation and uptake rates using a micro oxygen chamber. The presence of inhibitors will influence O_2-liberation rates.

4. *In vivo* chlorophyll *a* fluorescence - Not all the light that reaches photosystem II is used for photosynthesis and part is released as fluorescence. The quantity depends on several factors among which the state of Q_A reduction and the overall physiological condition are the most important. By exposing algae to toxic substances, the fluorescence peaks will vary according to the effect that the substance might have on the physiological condition of the algae. These reactions are extremely rapid and it should be possible to detect toxic substances within minutes.

5. Zooplankton toxicity tests - Various invertebrates are used in toxicity tests and, although easy to perform, they are time consuming. *Daphnia pulex* was used as test organism, and the results also served as control for the algal toxicity tests.

Four different parameters were used to determine algal growth rates as part of the algal growth potential procedure in the presence of potentially toxic compounds namely; cell numbers, dry weight, chlorophyll *a* measured fluorometrically and spectrophotometrically. Copper, cadmium and atrazine were found to be highly toxic, whilst mercury, phenol and gusathion showed little or no toxicity. The results showed that the time of exposure was very important, where on the one hand the algae adapted to the toxin indicating a lower toxicity, or on the other hand long exposures indicating high toxicity. The depletion of nutrients or the inability to distinguish between living and dead cells could influence long-term exposures,

which would have a direct bearing on the interpretation of results. We recommend short-term exposures and selection of the appropriate growth parameter for maximum sensitivity.

The major advantage of using *in vivo* chlorophyll *a* fluorescence is that measurements could be made within seconds and with relative ease. However, only one of the substances that was tested influenced chlorophyll *a* fluorescence markedly, namely the herbicide atrazine. It was concluded that although heavy metals (at the concentrations tested) and treated sewage effluent had no effect on *in vivo* fluorescence, further experimentation should be undertaken.

A short term small volume ^{14}C-assimilation algal toxicity test using *Selenastrum capricornutum* as test organism, proved to be a potentially valuable method. Generally the sensitivity of the method increased with increasing exposure time to the toxins. It was found that the method complied with the prerequisites of an acute test, because in most instancies toxicity could be detected within 30 min exposure.

O_2-production using either *Selenastrum* or *Chlorella* as indicator organisms to assess the presence or not of toxic compounds, was measured in a small volume oxygen chamber. These measurements were done at predetermined I_k irradiancies. It was shown that the heavy metals Hg, Cd and Cu and atrazine influenced the photosynthetic rates but the organophosphate gusathion had no effect. The oxygen evolution assay may be useful as a rapid preliminary screening method for the presence or not of toxic substances.

The acute toxicity of selected toxic substances were evaluated using *Daphnia pulex* as test organism. The herbicide, atrazine, had no effect whilst copper, cadmium, mercury, gusathion and phenol showed variable response. Gusathion, an insecticide, proved to be the most toxic of all the substances with LC50 values of 0.005 and 0.003 mg l^{-1} after 24 and 48 h exposure, respectively. The daphnid method was found to be reliable, repeatable and suited for the determination of water quality limitations.

When comparing the results from the algal assay procedures with that of the *Daphnia* mortality test it was found that the responses to the different compounds varied markedly between the different assay methods. Gusathion could only be detected with the *Daphnia* test, whilst atrazine could only be detected with the algae. It was *inter alia* conclude that much more research was needed before all the possibilities of using algae as test organisms were exhausted.

Based upon the results the following recommendations are made;

1. The ^{14}C-uptake method, because of its rapid response, should be used for screening purposes. Incubating at I_k irradiancies in the Photosynthetron allows for many samples to be processed at the same time, while working with small volumes of algal material.
2. *In vivo* chlorophyll *a* fluorescence showed potential as a means of testing for toxic substances and needs to be investigated further.
3. Further research should be carried out on algae to investigate various metabolic pathways not yet included in toxicity measurements and other indicators of the physiological state of the test organisms.
4. That more than one level in the trophic chain be used when investigating potentially toxic compounds or environmental quality.
5. That experimental protocols be standardized and if possible simplified to restrict experimental variations and error.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Historical perspective.

Over 2000 years ago, Aristotle placed freshwater animals in sea-water and observed their response, presumably to answer the question: How does this material affect this organism? In so doing, he conducted an aquatic toxicity test. Whether or not a substance affected an organism was relegated to the curiosity of physiologists, until a formal discipline known as toxicology, arose in the early 1800's in response to the development of inorganic chemistry (Zapp, 1980). During this initial phase man synthesized chemicals and then tested their effects on organisms. It was soon discovered whether these new compounds were poisonous or if they had no immediate effect. Although the adverse effects of certain compounds on organisms were known for a long time, the potential toxicity effects of substances, using fish as indicator organisms, were only done in the 1940's to 1950's. By that time, biologists had observed striking differences in aquatic populations living in streams that received waste materials from urban areas and those from natural areas. These changes in the invertebrate fauna led to the first attempts to formulate management strategies, in order to curb the impact of these hazardous compounds.

While surveys of organisms in streams documented adverse impacts of certain effluents, preventative measures required a quantification of the perturbation. Hart *et al.* (1945), Doudoroff *et al.* (1951), Cairns (1957), Henderson (1957), Tarzwell (1958), Sprague (1969, 1970, 1973), and others advocated, and demonstrated, the use of fish for detecting toxic substances in industrial effluents and to predict potential damage to streams, should spillage occur. The use of bioindicators became more common and was eventually broadened to include not only fish, but invertebrates and algae (Buikema *et al.*, 1982).

A bioassay could, therefore, be defined as a procedure that uses living organisms to estimate potential chemical toxic effects. In ecotoxicology bioassays are used to predict the levels of chemicals that produce no observable adverse effects upon populations, communities, and ecosystems and to identify biological resources at risk (Cairns and Pratt, 1989).

1.2 Bioassay objectives.

Bioassays are done to determine one or another biological response to unknown or known compounds and are usually carried out as a means of determining the concentrations of chemicals in the environment which would have no-adverse-biological effects. Bioassays may also be carried out to determine the effectiveness of a particular waste treatment process to reduce the toxicity of an effluent, or to determine why mixtures of chemicals act differently than one would expect from the individual components.

Although these exercises are ultimately related to the environment, they do not necessarily require direct extrapolation of effects to natural systems. The majority of bioassays, however, are carried out with the assumption that the test organisms are surrogates for the larger body of organisms comprising ecosystems. Basically, bioassays are conducted with the intention to predict harm or no harm after exposure of living organisms to certain concentrations of a chemical, or mixtures of chemicals, for certain periods of time (Cairns and Pratt, 1989).

Tebo (1985) summarized the objectives of bioassay tests used by the U.S. Environmental Protection Agency for establishing chemical limits and is quoted by Cairns and Pratt (1989) as follows:

"Screening:
- Tests should be rapid and inexpensive and should have wide applicability. Response should have high sensitivity to stress so that there will be low possibility of false-negatives.

Establishing Limitations:
- Tests should be directly related to environmental exposures and should be applicable to a wide range of site-specific situations.
- The response should be directly related to environmental hazard and should be easy to interpret and meaningful to the public and courts.
- Outputs should be directly translatable into specific decision criteria.
- To avoid possibilities of varying interpretation, it is preferable that the end point be a discrete variable. If the end point is not a discrete variable (such as death), justifiable decision criteria should be provided.

Monitoring:
- Tests should be rapid, inexpensive, and of known precision. Response should be sensitive and preferably related to the type of limitation imposed.

The desirable attributes of tests used for regulatory purposes are a function of these objectives."

1.3 Bioassay design.

In designing bioassays a distinction should be made between acute toxicity tests and chronic toxicity tests.

Acute toxicity tests provide information about the concentration of pollutants that would be severe enough to rapidly induce an effect over the short term and give mostly the relative lethality. The critical concentration is estimated by exposing organisms to a graded, logarithmic concentration range of waste or toxin and then observing their responses within seconds and up to 96 hours. Acute tests are considered ecologically significant, they are also scientifically and legally defensible, modest in predictive capability as well as simple and cost effective. These tests, therefore, are considered to have the greatest utility.

Chronic toxicity tests are mostly designed to provide information on the cumulative effect of various concentrations of toxicants on the survival, growth and reproductive success of an organism. The results reflect the consequences of long-term exposure to hazardous compounds. The reference concentration, as estimated by the chronic test, is a measure of a "safe" concentration, also known as the "no-effects" concentration. In other words, it is the highest exposure concentration that does not result in significant harm to the test organism in terms of survival, growth, or reproduction or it is interpolated as the geometric mean of the lowest concentration having an effect and the highest concentration of having no effect. The duration of chronic tests ranges from days for algae or protozoans, to weeks for small invertebrates, to months and over a year for larger invertebrates and fish (Buikema *et al.*, 1982).

The best developed test methods (in terms of standardization) are acute bioassays. Acute toxicity tests are useful (Stephan, 1982) because they establish initial benchmark or relative toxic potentials. They are ecologically relevant as they can be compared to, or calibrated with observations in the real world. They are reproducible, rapid and are easily interpretable (Sprague, 1969). As such, they are a logical starting point when attempting to understand the hazards of toxic materials to aquatic ecosystems. Ideally such tests would only be the first step in a comprehensive assessment process. It should also be pointed out that acute toxicity tests provide information on the relative toxicity among toxicants and species and were not designed to and cannot substitute chronic tests (Giesy and Graney, 1989).

1.3.1 Selection of test organism.

Most toxicity tests have evaluated the short term lethality of wastes to adult fish. Fish are the most popular test organism because they are presumed to be the best understood organism in the aquatic environment and are perceived as most valuable by the majority of laymen. However, when wastewater flows into an aquatic ecosystem, it influences

hundreds of species, even when the discharge area is very small. Patrick *et al.* (1968) and others have demonstrated that diatoms and macroinvertebrates are often more sensitive to toxicants than fish, and they represent a greater portion of the biomass in natural systems than do fish. Fish also depend on aquatic plants for cover and for spawning. Thus, even if the goal is the protection of fish alone, testing and protecting other environmental members is necessary.

Because of this, many toxicity tests with organisms other than fish (e.g. invertebrates, algae and aquatic plants) have become increasingly common or are being proposed. Additionally, tests with various ages and life stages of all organisms are used because younger animals are often more sensitive to toxicants than adults (Cairns *et al.*, 1965). Furthermore, protocols differ in their guidance for selection of test species, e.g. indigenous versus readily available laboratory cultured species, and so on. Consequently careful consideration is required before deciding on which test organism to use.

In their preproposed guidance for premanufactured tests, the United States Environmental Protection Agency (US EPA, 1979) published a variety of tests for determining the potential impact of new chemicals. Selection of test organisms was based on four criteria:

1. The organism must be representative of an ecologically important group (in terms of taxonomy, trophic level or realised niche).
2. The organism should occupy a position within a food chain leading to man or other important species.
3. The organism must be widely available, be amenable to laboratory testing, easily maintained, and genetically stable so that uniform populations can be used in tests.
4. There must be adequate background data available on the organism (i.e. its physiology, genetics, taxonomy, and its role in the natural environment must be understood).

A complete assessment of the presence of contaminants in aquatic ecosystems is basically achieved by using organisms from various trophic levels in comparative bioassays (Gächter, 1979; Maciorowski *et al.*, 1981; Bringmann and Kuhn, 1980; Munawar *et al.*, 1989).

1.3.2 Phytoplankton assay techniques.

The phytoplankton, together with other green plants, are the primary producers in aquatic ecosystems and, as such, are at the base of aquatic food chains. Because of this, they are ideal for use in bioassays that predict and determine the potential effects of substances on aquatic food chains. Furthermore, the use of algae as test organisms is gaining support due to their structural simplicity, ubiquitous abundance in nature, and the ease of obtaining commercially available cultures for laboratory testing (Munawar *et al.*, 1989). Also, algal bioassays directed towards toxicity tests are rapid, inexpensive, sensitive, and can effectively be used to assess toxic substances which are found in concentrations too low for effective detection by organisms of higher trophic levels (Munawar and Munawar, 1987; Wong and Couture, 1986).

Phytoplankton in their natural environments, unlike many other organisms, are affected both by nutrients and contaminants. A large variety of physiological and anabolic tests are available to choose from depending on the type of problem, precision required, and budget. Several laboratory techniques are available and include *in vitro* batch and continuous cultures. Details of these techniques are provided by Málek and Fencl (1966), Miller *et al.*, (1978) and others.

The use of natural phytoplankton is favoured over laboratory-grown cultures by Munawar *et al.*, (1989), because extrapolation of laboratory data to natural conditions are often difficult and misleading. For example, toxicity tests using natural assemblages compared to laboratory-grown cultures have yielded results showing enhancement of primary productivity in natural phytoplankton, while the same test indicated inhibition in mixed cultures (Munawar and Munawar, 1987).

However, Lewis (1990), concluded that; "Laboratory-derived algal toxicity tests are worth the effort since they are necessary for the complete safety evaluation of chemicals and effluents. Currently, these data are seldom the 'driving force' in regulatory assessment because of the limited data available and in some cases to the apparent lack of sensitivity of the usual one algal species exposed to the toxicant. There is a need, therefore, to increase the quality and quantity of laboratory-derived toxicity data for algae since there is little technical evidence that animal test data should be substituted. The additional data for algae will result most likely from use of one of the several existing standard test methodologies. Improvements to these methods in the short-term by using several test species and in the long-term by establishing the biological significance of the inhibitory and stimulatory effect levels will increase the use of these data in the safety assessment process. Overall, these improvements should receive high priority by the scientific community and be recognized as needed by the regulatory community."

Several new techniques have been developed such as Video Analysis Systems, *In situ* Plankton Cages, Limited Sample Bioassays, Epifluorescence Microscopy and Flow Cytometry (Munawar *et al.*, 1989; Munawar and Munawar, 1987; Weisse, 1989; Berglund and Eversman, 1988). These new techniques, together with a wide variety of existing procedures and computer-assisted methodologies, provide an excellent array of tests to assist in environmental protection and conservation of endangered aquatic environments.

1.4 Application of toxicity data in environmental management.

The goal in the design and use of biomonitoring toxicity tests, is to be able to predict with accuracy a certain concentration that will not harm an entire system in a responsible and cost effective manner. At present, even with the most favourable single species toxicity tests, the problem remains of predicting the possible harmful effects of a compound or combination of compounds to a complex system (Buikema *et al.*, 1982).

1.5 Aims of this study.

Although effluent standards (See Chapter 2, Table 2.2) are at present based only on chemical and physical parameters, the results obtained from the monitoring of effluents and receiving waters may indicate the desirability of including biological parameters (DWA, 1986). Bioassays are necessary in water pollution evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota (Tarzwell, 1958). Both biological and chemical evidence is needed to assess risk to ecosystems effectively. The ability to detect a compound does not ensure that biological effects can be predicted and the failure to detect a released chemical does not preclude its effects (Cairns and Pratt, 1989). Different kinds of aquatic organisms are also not equally susceptible to the same toxic substances (APHA, 1989).

The objectives of this study were, therefore:
1. To use physiological indicators of algae to determine the presence of toxic substances.
2. To compare the response of algae to commonly used zooplankton toxicity tests.
3. To possibly develop a bioassay method which is cost effective and reliable.
4. To make recommendations as to which toxicity bioassay methods are the best suited to detect specific compounds.

In order to achieve this, the following, five different basic procedures were evaluated with six known toxic substances as well as secondary treated municipal sewage effluent (See Chapter 2).

1. Algal Growth Potential (AGP) - This method has been in use for many years to indicate biostimulation and toxicity (Miller *et al.*, 1978). Although it is time consuming it was used as a reference for comparative purposes.

2. ^{14}C-uptake rates by algae - This method is based on the techniques used to measure phytoplankton primary productivity. From the ^{14}C-uptake rates different algae and

conditions could be compared and the presence of inhibiting factors and it should be possible to show the presence, or not, over a relatively short period.

3. O_2-evolution rates - Photosynthetic rates could also be measured in terms of oxygen liberation and uptake rates using a micro oxygen chamber. The presence of inhibitors will influence O_2-liberation rates.

4. *In vivo* chlorophyll fluorescence - Not all the light that reaches photosystem II is used for photosynthesis and part is released as fluorescence. The quantity depends on several factors among which the state of Q_A reduction and the overall physiological condition are the most important. By exposing algae to toxic substances, the fluorescence peaks will vary according to the effect that the substance might have on the physiological condition of the algae. These reactions are extremely rapid and it should be possible to detect toxic substances within minutes.

5. Zooplankton toxicity tests - Various invertebrates are used in toxicity tests and, although easy to perform, they are time consuming. *Daphnia pulex* was used as test organism, and the results also served as control for the algal toxicity tests.

CHAPTER 2

GENERAL MATERIAL AND METHODS

The following material, methods and growth conditions were used throughout this study except where otherwise indicated.

2.1 Cultures and growth conditions.

2.1.1 Test organisms - Unialgal cultures of *Selenastrum capricornutum* (CCAP 278/4) and *Chlorella vulgaris* (CCAP 211/12) were obtained from the Culture Collection of Algae and Protozoa (CCAP), Natural Environment Research Council, Cambridge, U.K. The reason for selecting these algae were because they maintain unicellularity throughout their life cycles which make them suitable for cell counting using electronic particle counters.

2.1.2 Growth medium - The algae were grown in Synthetic Algal Nutrient Medium (SANM) (Table 2.1), (Miller *et al.*, 1978). The culture medium was prepared by adding one ml of each stock solution, in the order given, to approximately 900 ml of distilled water and then made-up to one litre with distilled water. The pH was adjusted to 7.5 ± 0.5 either with 0.1 N NaOH or HCl as required.

2.1.3 Growth conditions - Semi-continuous cultures were maintained in a Conviron Model E7H (Controlled Environments, Winnipeg, Canada) growth cabinet at 23 ± 2 °C. Continuous light was supplied at 300 µmol quanta m^{-2} s^{-1} by cool white fluorescent tubes and incandescent lamps arranged alongside and above cultures. The medium was well buffered because the pH remained at 7.5 ± 0.5 even after continuously aerating with air and the continuous bubbling kept the cells in suspension.

Cells in the exponential growth phase (about 10^4 cells ml^{-1}) were used in the experiments. The exponential growth phase was determined from daily cell counts with an electronic particle counter (Coulter Multisizer II, Coulter Electronics, England) and the exponential growth phase was determined from a graphical plot.

Table 2.1 Synthetic Algal Nutrient Medium for freshwater algae (Miller *et al.*, 1978).

Stock solutions	Amount per 500 ml
1. $NaNO_3$	12.750 g
2. $MgCl_2.6H_2O$	6.082 g
3. $CaCl_2.2H_2O$	2.205 g
4. Micronutrient stock solution:	
H_3BO_3	92.760 mg
$MnCl_2.4H_2O$	207.69 mg
$ZnCl_2$	1.635 mg
$FeCl_3.6H_2O$	79.880 mg
$CoCl_2.6H_2O$	0.714 mg
$NaMoO_4.2H_2O$	3.630 mg
$CuCl_2.2H_2O$	0.006 mg
$Na_2EDTA.2H_2O$	150.00 mg
5. $MgSO_4.7H_2O$	7.350 g
6. K_2HPO_4	0.522 g
7. $NaHCO_3$	7.500 g

The various toxic substances and their concentration ranges used in the tests, are listed in Table 2.2. The effluent quality standards and criteria applicable to direct discharges into

fresh water sources laid down by law and environmental protection agencies, which are also given in this table, served as guidelines for the concentrations used in the present tests. The way in which these substances were administered depended on the particular experiment and are discussed with each experiment (Chapters 3, 4, 5, 6 and 7).

2.2 Toxic compounds used in toxicity tests.

2.2.1 Cadmium (Cd^{++}) - It is a soft, white, easily fusible metal similar to zinc and lead, in many properties, and readily soluble in mineral acids. Biologically cadmium is a nonessential, nonbeneficial element recognized to be of high toxic potential. It is deposited and accumulated in various body tissues and is found in varying concentrations throughout all areas populated by man. Within the last three decades industrial production and the use of this metal have increased. Cadmium may function in or may be an etiological factor for various human pathological processes including testicular tumors, renal disfunction, hypertension, arteriosclerosis, growth inhibition and chronic diseases of old age.

Cadmium occurs in nature mainly as a sulphide salt, frequently in association with zinc and lead ores. Accumulations of cadmium in soils in the vicinity of mines and smelters may result in high local concentrations in nearby waters. The salts of the metal may also occur in wastes from electroplating plants, pigment (paint) works, textile and chemical industries.

2.2.2 Copper (Cu^{++}) - Copper occurs as a natural or native metal in various mineral forms such as cuprite and malachite. The main copper ores are sulphides, oxides and carbonates. Copper has been mined and used in a variety of products by man since prehistoric times. Oxides and sulphates of copper are used for pesticides, algicides and fungicides. Copper is frequently incorporated into paints and wood preservatives to inhibit the growth of algae and invertebrate organisms.

Table 2.2. Toxic compounds used in the tests and their concentrations in accordance with effluent standards and criteria, applicable to direct discharges into fresh water sources, as laid down by law and environmental protection agencies. Note that the concentrations given are for the metals and not for the salts. General standards are applied universally while special standards are applicable to specified streams.

Compound	Concentration ($mg\ l^{-1}$)	Standard ($mg\ l^{-1}$)	
		General[1]	Special[1]
Cadmium as $CdCl_2.H_2O$	0.005, 0.01, 0.05, 0.1, 0.25, 0.5 and 1	0.05	0.05[1]
Copper as $CuCl_2.2H_2O$	0.002, 0.01. 0.02, 0.1, 0.2, 1, 2 and 5	1.0	0.20[1]
Mercury as $HgCl_2$	0.002, 0.005, 0.01, 0.02, 0.05 and 0.1	0.02	0.02[1]
Atrazine an organochloride.	0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5	0.005	0.005[2]
Gusathion or Azinphos-methyl, an organophosphate.	0.00001, 0.0001, 0.001, 0.01, 0.1 and 1	0.01	0.01[3]
Phenol	0.001, 0.01, 0.1, 1, 10 and 100	0.10	0.01[1]

[1] Article 21, Water Act, 1956
[2] Bayer SA (Pty) Ltd (personal communication)
[3] U.S. EPA (1976), (in $\mu g\ l^{-1}$)

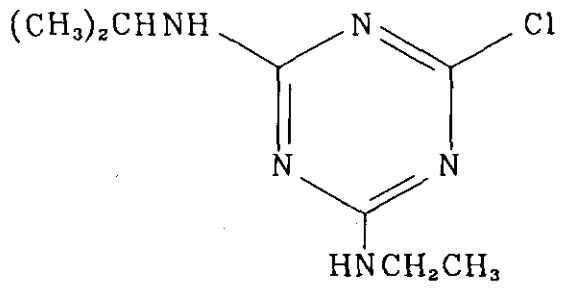
Copper is an essential trace element for the propagation of plants, performs vital functions in several enzymes and plays a major role in the synthesis of chlorophyll. Copper is required in animal metabolism. It is important in invertebrate blood chemistry especially the synthesis of hemoglobin. In some invertebrate organisms a protein, hemocyanin, contains copper and serves as the oxygen-carrying mechanism in the blood.

An overdose of ingested copper acts in mammals as an emetic. Prolonged oral administration of excessive quantities of copper may result in liver damage. Copper sulphate has been widely used in the control of algae in water supply reservoirs and recreational lakes. The toxicity of copper to aquatic life is dependent on the alkalinity of the water because the copper ions are complexed by anions, which in turn affect its toxicity. At lower alkalinity copper is generally more toxic to aquatic life. Other factors affecting toxicity include pH and organic compounds.

2.2.3 Mercury (Hg^{++}) - Mercury is a silver-white, liquid metal that solidifies at -38.9 °C to form a tin-white, ductile malleable mass. Mercury is widely distributed in the environment and biologically is a non-essential or non-beneficial element. Historically it was recognized to possess a high toxic potential and was used as germicidal or fungicidal agent for medical and agricultural purposes.

Mercury intoxication may be acute or chronic and toxic effects vary with the form of mercury and its mode of entry into the organism. The mercurous (Hg^{+}) salts are less soluble than the mercuric (Hg^{++}) and consequently less toxic. Symptoms of acute, inorganic mercury poisoning include pharyngitis, gastro-enteritis, vomiting followed by ulcerative hemorrhagic colitis and nephritis.

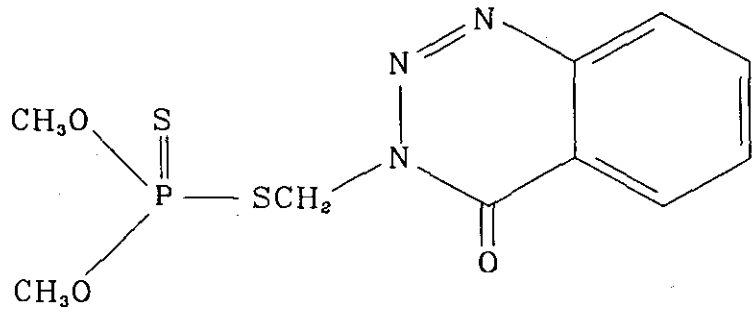
2.2.4 Atrazine [6-Chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine] ($C_8H_{14}ClN_5$) -



Atrazine is used as a selective herbicide and is also known under the names; AAtrex, Atranex, Gesaprim and Primatol A. This commonly used herbicide inhibits photosynthesis by blocking electron transport within the Hill reaction of photosystem II (Moreland, 1980). This organochloride is soluble in water at 25 °C at a concentration of 70 mg l^{-1} and in methanol at 18 g l^{-1}. It is hydrolyzed to an inactive hydroxy derivative by alkali or mineral acids. Inhalation hazard is low in humans and symptoms are adrenal degeneration, congestion of the lungs, liver and kidneys.

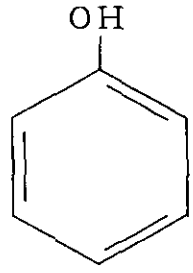
2.2.5 Gusathion {Phosphorodithioic acid O,O-di-methyl S-[(4-oxo-1,2,3-benzotriazin-3(4H)-yl)methyl]ester} ($C_{10}H_{12}N_3O_3PS_2$) -

Gusathion is an organophosphate used as an insecticide and acaricide and is also known under the names; Azinphos-methyl, Cotnion-methyl and Guthion. This substance is soluble in water at 25 °C at a concentration of 33 mg l^{-1}. It is also soluble in methanol, ethanol, propylene glycol and other organic solvents. It hydrolyzes in acid or cold alkali.



Organophosphate pesticides are toxic because they inhibit the enzyme acetylcholinesterase (AChE) which is essential to nerve impulse conduction and transmission. Although cumulative inhibition of brain AChE and mortality of fish were shown after repetitive injection with gusathion (Benke and Murphy, 1974), there is no evidence to indicate that gusathion would cause adverse effects through the food chain.

2.2.6 Phenol (C_6H_6O)



Phenolic compounds include a wide variety of organic chemicals. The phenols may be classified into monohydric, dihydric and polyhydric phenols depending upon the number of hydroxyl groups attached to the aromatic ring. Phenol itself, which has but one hydroxyl group, is the most typical of the group and is often used as a model compound. Phenolic compounds arise from the distillation of coal and wood, from oil refineries, chemical plants, livestock dips, human and other organic wastes, hydrolysis, chemical oxidation, microbial degradation of pesticides and from naturally occurring sources and substances.

Because of the high oxygen demand of the compounds it can affect freshwater fish by lowering the amount of available oxygen. Various environmental conditions will increase the toxicity of phenol. Lower dissolved oxygen concentrations, increased salinity and increased temperature all enhance the toxicity of phenol. A major aesthetic problem associated with phenolic compounds is their organoleptic properties in water.

Analytical grade cadmium ($CdCl_2.H_2O$), copper ($CuCl_2.2H_20$), mercury ($HgCl_2$) and phenol (C_6H_6O) were obtained from Merck Chemicals (Pty) Ltd. Agricultural grade atrazine (BAC Atrazine 500FW) and gusathion (Gusathion 35WP) were kindly provided by Bayer SA (Pty) Ltd, Bloemfontein.

Tertiary treated municipal sewage effluent was collected from the Bloemfontein Municipality Sewage-works, just before its discharge into Bloemspruit. This is a spruit feeding the Modder river from which water is abstracted to be purified for domestic use. The Water Act, 1956, provides for the introduction of standards (Table 2.2) relating to the quality of receiving streams or to the quality of effluent that is discharged into fresh water sources. Since our toxin concentrations were based on these standards it was decided to monitor treated effluent instead of raw sewage.

The effluent was filtered, after collection, through 0.45 µm Whatman GF/C glassfibre filters to get rid of all the suspended material and stored at 4 °C until used in the assays. Of this filtered effluent the following concentration series were made up; 100, 75, 50 and 25% using either SANM in the case of algal assays (Chapters 4, 5 and 6) or moderately-hard reconstituted water for the zooplankton assays (Chapter 7). Incubation with algae was always done in a ratio of 1:10.

2.3 Calculations and data presentation.

When interpreting results from microbial toxicity tests with continuous, quantitative response, one should ideally derive a relationship between the relative response, *p*, and the concentration, *c*, or log concentration of the toxicant, log *c*. This relationship is used inversely to estimate certain characteristic toxicant concentrations (EC values = effective concentration values or LC values = lethal concentration value) that would inhibit/kill a certain percentage of the test organisms, such as EC/LC10, EC/LC50 and EC/LC90 for 10, 50 and 90 % inhibition/mortality which relates to a relative response of 0.9, 0.5 and 0.1 respectively. The response variable could be either the calculated average growth rate (Chapter 3), the index of toxicity calculated from *in vivo* fluorescence (Chapter 4), ^{14}C-uptake (Chapter 5) or the measured oxygen evolution (Chapter 6).

The data were first normalized by dividing with the controls (average of 2-3 replicates);

$$p = r / r_o \qquad (1)$$

where r = the measured response, r_o = the control response and p = the relative response.

The percentage inhibition (q) is then calculated from equation (1) as follows;

$$q = (1 - p) \times 100 \qquad (2)$$

After normalization, dose-response curves were obtained from non-linear least square fits of the data using a four parameter logistic equation (Nyholm *et al.*, 1992);

$$y = a / [1 + \exp^{b(z - c)}] + d \qquad (3)$$

where,
z = log (concentration) (mg l^{-1}) (more symmetric curves were obtained by using log *c* instead of *c*)
a = response range
b = slope coefficient
c = inflection point of curve
d = minimum response

The EC (effective concentration) estimates are calculated from the non-linear least square fits using the regression curve inversely. With the logistic equation, estimating EC10 (concentration/dose causing a 10 % inhibition) the inverse function is;

$$\log(EC10) = \{\ln [(a / 0.9 - d) - 1] / b\} + c \qquad (4)$$

for EC50 (50 % inhibition);

$$\log(EC50) = \{\ln [(a / 0.5 - d) - 1] / b\} + c \qquad (5)$$

and for EC90 (90 % inhibition);

$$\log(EC90) = \{\ln [(a / 0.1 - d) - 1] / b\} + c \qquad (6)$$

CHAPTER 3

THE USE OF *SELENASTRUM CAPRICORNUTUM* GROWTH POTENTIAL AS A MEASURE OF THE TOXICITY OF A FEW SELECTED COMPOUNDS.

Introduction

In recent years, considerable effort has been devoted towards developing standardized procedures and guidelines for assessing the presence of toxic substances and tests using the growth potential of algae (AGP = algal growth potential). Conducting algal tests on a routine basis and working out standard procedures and test guidelines are not merely a question of understanding algal growth phenomena. There are also questions of compromising between practical considerations (cost and simplicity) and scientific preferences. It is desirable that such compromises be made on sound scientific terms, which involves a thorough understanding of how algal test systems can be manipulated and how various experimental factors may influence the results (Nyholm and Källqvist, 1989).

Test organisms for routine use should, first of all, be good laboratory organisms rather than species that are very sensitive or very abundant in nature. The widely used freshwater green alga *Selenastrum capricornutum* is such a species. Among the green algae, this species seems to have a "medium sensitivity" (Walsh and Merrill, 1984), and is easy to culture. It has a very characteristic shape, so that contamination of cultures is easily detected, and it is suitable for cell counting by means of electronic particle counters.

The composition of the test medium may influence the assessment of toxicity considerably. For example Turbak *et al.* (1986) observed differences in the results of experiments conducted in a standard algal growth medium and in nutrient-enriched natural water samples, suggesting that water chemistry plays an important role in the determination of toxicity thresholds for algae. Adams and Dobbs (1984) indicated that the composition of the growth medium has a significant effect on the rate of growth and maximum cell yields and that changes in concentrations of nitrogen and phosphorus affect toxic limits. They also pointed out that trace nutrients may also influence the results. With regard to general-purpose synthetic media, as used in standardized tests, the concentration of chelators may be critical in particular when testing for heavy metals. This is because a chelating agent, even at low concentrations, adsorbs and releases trace elements rendering them available. A very useful medium is, therefore, the Synthetic Algal Assay Medium (SANM) of Miller *et al.*, 1978, which contains 300 $\mu g\ l^{-1}$ $Na_2EDTA.2H_2O$ in combination with 160 $\mu g\ l^{-1}$ $FeCl_3.6H_2O$.

Little is known about how irradiance may interact with toxicity. It is possible, for instance, that specific effects on photochemical reactions are expressed sooner at low irradiancies. Accounting for such phenomena are, however, considered beyond the scope of routine testing. The question might arise as to whether a light dark cycle or continuous illumination should be used. From a practical viewpoint, continuous irradiance presents a great advantage in routine testing, as constant growth conditions and unsynchronized algal populations can be maintained. Most green algae grow well in continuous irradiance and most test protocols with green algae, (ISO, 1987; OECD, 1984; Miller *et al.*, 1978; US EPA, 1982) prescribe continuous irradiance and it is, therefore, recommended.

Standardize tests are normally carried out at temperatures somewhat below the optimum for a particular test species (23 - 24 °C for *Selenastrum* [ISO, 1987; OECD, 1984; Miller *et al.*, 1978]). Although it is relatively easy to control temperature, amongst the test flasks, it is more important to ensure that the temperature is uniform than to control the temperature at a fixed level.

In an experiment conducted by Nyholm and Källqvist (1989) comparisons of the pH's were made between cultures which were continuously shaken and those shaken by hand once a day. In the continuously shaken cultures, pH differences were marginal. In the static cultures, the pH starts to increase to high levels after about two days. EPA algal assay

medium for freshwater algae (Miller *et al.*, 1978) has a bicarbonate content of 15 mg l^{-1} $NaHCO_3$. At equilibrium with air, this results in a pH of 7.5. Further means of controlling the pH is by terminating the test after a short period (e.g. after 2 to 3 days) or by inoculating with low cell densities.

It has become clear in recent years that a general-purpose standard toxicity test should preferably be of relatively short duration and restricted to the initial period of exponential growth, lasting 2 to 4 days (Walsh *et al.*, 1982; Nyholm and Källqvist, 1989). During the 1970's, algal assays were widely used as growth potential tests to assess the nutritional status of natural waters (US EPA, 1971). Toerien *et al.* (1971) in determining provisional algal assay procedures stated that batch culture assays appear to be best suited for screening and routine monitoring purposes. The endpoint in these types of assays is usually the ultimate algal biomass or yield as reached after 14 days (Miller *et al.*, 1978). This final yield may be influenced by the presence of toxicants, but for most applications it does not provide a good endpoint for toxicity tests. The reasons for this are: (1) even with considerable reductions in the growth rate the final yield will not be affected, because the toxicant-affected cultures may gradually "catch up" with the controls when nutrients become limiting; (2) in the duration of the test, toxicity can be lost due to various mechanisms and thus have little or no effect on the final yield (Walsh *et al.*, 1982).

There appears to be a general consensus that for most purposes algal toxicity tests should be of relatively short duration and terminated well before the growth of the control cultures becomes severely depressed during the stationery phase, due to limiting factors. Accordingly, the position of the ISO (International Standards Organization) and the OECD (Organization for Economic Cooperation and Development) are that tests should be designed so that control cultures grow exponentially for the entire duration of the test (Nyholm, 1985). Results are most easily interpreted if there is no lag phase in the control cultures, a condition that can be ensured by inoculating with exponentially growing cells propagated under the test conditions.

After inoculation in fresh nutrient medium some time elapses before the number of algae begin to increase. This period is termed the "lag phase" and the length thereof depends mainly on the age and type of inoculum. The next stage represents the beginning of cell division and multiplication which is the beginning of the exponential or logarithmic growth phase in which the algae multiply uniformly at a constant growth rate. The rate of growth during this phase is characteristic for any given alga under the specific set of conditions and represents the maximal reproductive capacity of that alga in the specific environment. Environmental factors that govern the rate of growth include the nature and concentration of the nutrients, pH, temperature, light, and other physical and chemical variables. Eventually, some nutrient becomes exhausted or the environment becomes less favourable to growth and the growth rate decreases to a stationery phase. The stationery phase of some algal cultures passes into a subsequent death phase where death and lysis of algae in the culture begins to exceeds growth (Toerien *et al.*, 1971).

The response obtained in most microbial toxicity tests is a quantitative, continuous variable. For example, the specific growth rate, i.e. the growth rate that is specific for an organism, environment and culture medium, could be measured as an increase in biomass. It could also be expressed in terms of an increase or decrease in dry weight, cell number or chlorophyll *a* concentration. Nyholm (1985) concluded, from a theoretical mathematical viewpoint, that some measure of the specific growth rate should be used as the response variable in algal growth inhibition tests, rather than the biomass at the end of the test because the specific growth rate is a function of the growth rate limiting nutrient, or toxin concentration, when all other factors are in excess. It was pointed out that, with exponentially growing cultures, EC values derived from biomass data were dependent on time as well as on the maximum specific growth rate, which was a characteristic of the particular test system.

It should be possible to estimate the EC50 concentration as well as adequately describe the initial concentration-response curve using statistical methods. Although many methods are equally suitable for calculating EC50 (Walsh *et al.*, 1987), there may be substantial

differences between methods with respect to their ability to describe the initial response curves and to reliably estimate such concentrations as the EC10 (Nyholm *et al.*, 1992)

Inhibitory effects in algal growth tests may be expressed in several ways and endpoints other than biomass or growth rate could be used, e.g. as dry weight, cell numbers or chlorophyll *a* concentration (determined spectrophotometrically or fluorometrically). The objective of this study was, therefore, to interpret toxicity values (concentrations) as obtained with other test methodologies such as short-term *in vivo* chlorophyll *a* fluorescence, ^{14}C-uptake and O_2-evolution of algae and longer term zooplankton mortality tests. Although controversy exists with regard to the choice of the response variable, the average growth rate (Nyholm, 1985), was chosen for the purpose of this study.

Material and methods

Test organism - The freshwater alga *Selenastrum capricornutum* (CCAP 278/4) was used for growth potential tests (See Chapter 2). Stock cultures of *Selenastrum* were maintained in the growth medium and under the conditions as described in Chapter 2.

Test conditions and procedures - Autoclaved Erlenmeyerflasks (125 ml) containing 50 ml medium were inoculated with *Selenastrum* under sterile conditions. The flasks were inoculated with exponentially growing cells to give a final concentration of approximately 10^4 cells ml^{-1}. The cultures were incubated at 23 ± 2 °C under continuous illumination provided from a rack of fluorescent tubes of either "cool white" or "gro-lux" color, vertically arranged below the cultures in a Conviron Model E7H (Controlled Environments, Winnipeg, Canada). Irradiance, measured with a Li-Cor Quantum Meter, Model LI-185B equipped with a LI-190SB quantum sensor, was 120 ± 24 µmol quanta m^{-2} s^{-1}. Flasks were handshaken daily to the keep cells in suspension and to aerate cultures.

Batch culture toxicity tests were conducted with six reference toxicants namely; copper, cadmium, mercury, atrazine, gusathion and phenol (See Table 2.2). The toxicants were added aseptically to the cultures as 1 ml volumes from stock solutions to give the desired final concentrations. The control flasks received 1 ml sterile distilled water instead of toxin. Immediately after the addition of a toxin, whilst stirring 10 ml of the cultures were withdrawn for analysis. Further samples were taken after 24, 48 and 72 h.

Analyses - For each flask growth rates were determined from increases in; i) dry weight, ii) cell numbers, iii) chlorophyll *a* content measured spectrophotometrically, and iv) chlorophyll *a* content measured fluorometrically.

Cellular volume and cell numbers were measured using a Coulter Multisizer II (Coulter Electronics, England), equipped with a 100 µm aperture tube. Dry weights were calculated from mean cell volumes (MCV) making use of a conversion factor of 3.6×10^{-7} (mg dry weight l^{-1})/[MCV (µm^3) cells ml^{-1})] for *Selenastrum*, established for exponentially growing cells (Miller *et al.*, 1978).

For chlorophyll *a* measurements, 5 ml of algal sample was centrifuged for 5 minutes at 3000 rpm. The supernatant was decanted and 10 ml 100 % methanol [Merck Chemicals (Pty) Ltd, Johannesburg] was added directly into the centrifuge tube with algal cells. Samples were then resuspended by vortexing. Chlorophyll *a* was extracted in hot methanol, and measured on a Philips PU8700 Series UV/Vis Spectrophotometer (Pye Unicam Ltd, Cambridge, England), (Sartory, 1982; Sartory and Grobbelaar, 1984).

The same extract was used for chlorophyll *a* fluorescence measurements using a Hitachi Model F-2000, Fluorescence Spectrophotometer (Hitachi, Ltd. Tokyo, Japan) against a standard curve of chlorophyll *a* (99 % pure, Sigma Chemical Co., St. Louis, Missouri). Maximum sensitivity for chlorophyll *a* extract was obtained at an excitation wavelength of 430 nm and an emission wavelength of 663 nm which was the same as reported by Abou-Waly *et al.*(1991b).

Calculations - The average growth rate was calculated as;

$$\mu_{av} = (\ln X_t - \ln X_o) / t$$

where X_o is the biomass at time zero and X_t represents the biomass of cultures at time t (Nyholm, 1985). The algal biomass was expressed in terms of i) dry weight, ii) cell number, iii) chlorophyll *a* concentration measured spectrophotometrically and iv) chlorophyll *a* concentration measured fluorometrically.

The average growth rate for each parameter was taken as the measured response, from which the relative response (p) [Chapter 2, Equation (1)] was calculated. Further calculations and treatment of data were done as described in Chapter 2.

Results

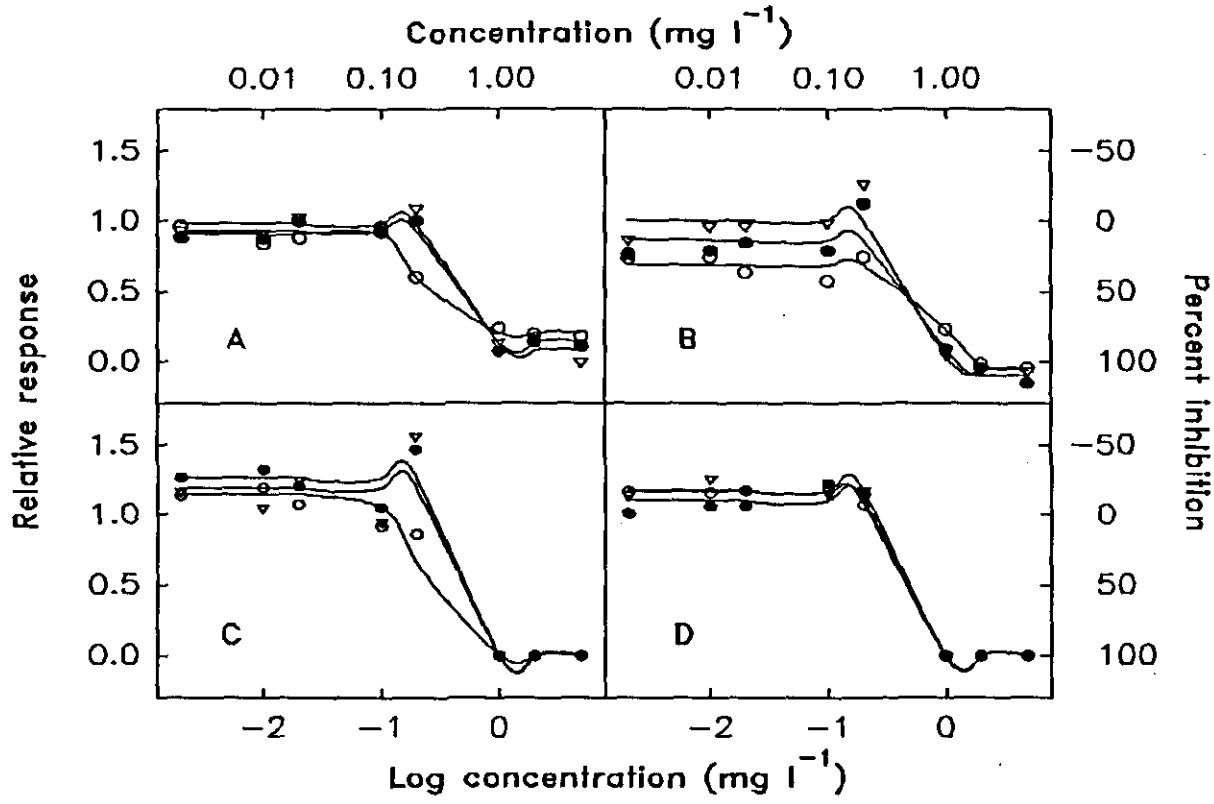


Fig. 3.1. The relative growth response of *Selenastrum capricornutum* to different concentrations of copper. Relative responses were obtained from changes in cell numbers (n ml^{-1}) (A), dry weight (mg l^{-1}) (B), chlorophyll *a* content (µg l^{-1}), measured fluorometrically (C) and spectrophotometrically (D).
(○ = 24h, ● = 48h and ▽ = 72h)

The relative response of *Selenastrum* to various concentrations of copper are shown in Figure 3.1. The results show that copper at a concentration of 1 mg l^{-1} completely inhibited *Selenastrum* growth as measured using all four growth indicators (Figures 3.1 A, B, C and D). Copper slightly stimulated growth at concentrations of 0.002 - 0.2 mg l^{-1} as indicated from the chlorophyll *a* content (Figure 3.1 C and D). In terms of cell numbers (Figure 3.1 A) some inhibition at a concentration of 0.2 mg l^{-1} was seen after 24 h. The inhibitory responses after 24 h are turned into stimulatory responses after 48 and 72 h of exposure. The stimulation after 48 and 72 h at a concentration of 0.2 mg l^{-1} was higher than the initial stimulation at concentrations below 0.2 mg l^{-1}.

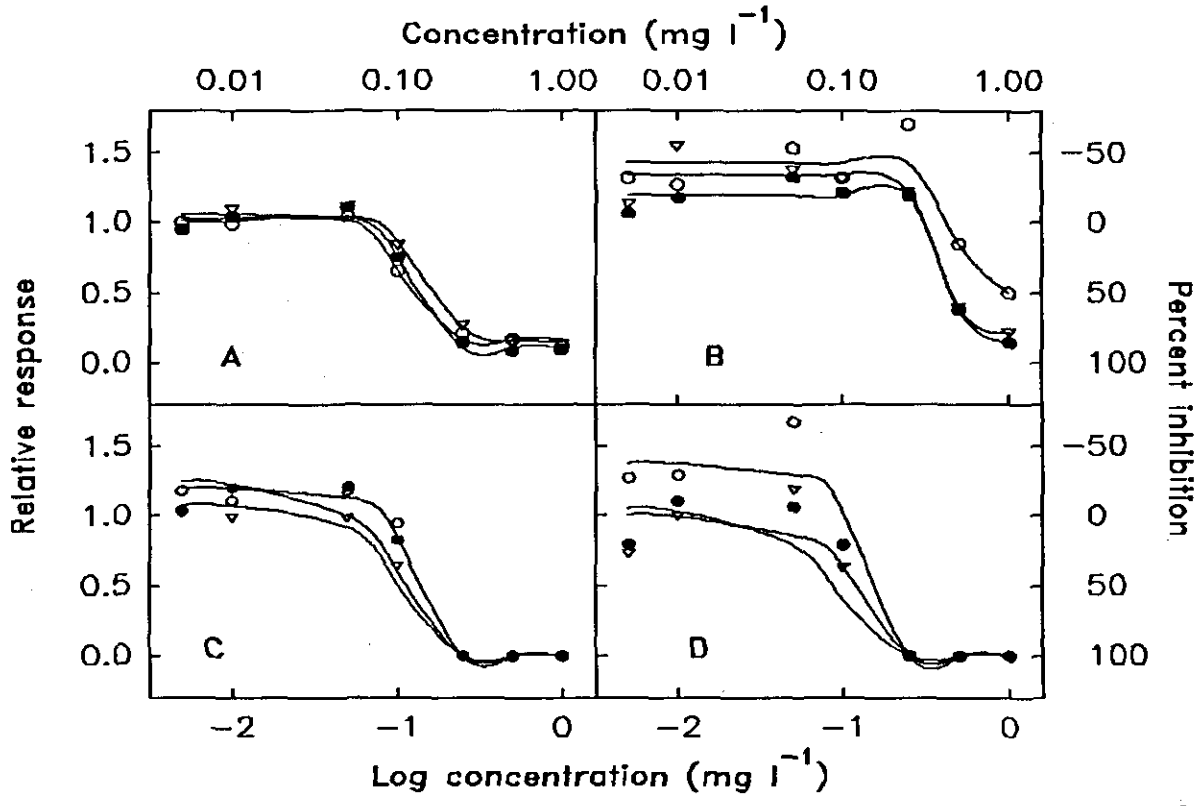


Fig. 3.2. The relative growth response of *Selenastrum capricornutum* to different concentrations of cadmium. Relative responses were obtained from changes in cell numbers (*n* ml^{-1}) (A), dry weight (mg l^{-1}) (B), chlorophyll *a* content (µg l^{-1}), measured fluorometrically (C) and spectrophotometrically (D). (o = 24h, ● = 48h and ▽ = 72h)

The relative response of *Selenastrum* to various concentrations of cadmium are shown in Figure 3.2. Enhancement of growth was observed at low concentrations of 0.005 and 0.01 mg l^{-1} cadmium, especially in terms of dry weight (Figure 3.2 B) and fluorometric chlorophyll *a* (Figure 3.2 D) where the stimulation was nearly 50 % (i.e. a relative response of 0.5) after 24 h of exposure. This stimulatory effect declined to that of the control after 48 and 72 h exposure (Figure 3.2 D). *Selenastrum* growth was increasingly inhibited with an increasing cadmium concentration between 0.05 and 1.0 mg l^{-1}. With a tenfold increase (0.1 - 1.0 mg l^{-1}) in the dosage the response varied from virtually no inhibition to 100 % inhibition. Both copper and cadmium were highly toxic as illustrated by the zero responses in the graphs (Figures 3.1 and 3.2).

The sensitivity of *Selenastrum* to mercury is variable (Figure 3.3) depending on the biomass parameter used. A relative response of 0.5 (50 % inhibition) was obtained with cell number (Figure 3.3 A) and chlorophyll *a* measurements (Figure 3.3 C and D) after 24 h exposure to mercury. After 48 h exposure only chlorophyll *a* measurements show a 50 % inhibition and after 72 h exposure no EC50 could be determined in the concentration range of 0.002 to 0.1 mg Hg(II) l^{-1}. The first observable (EC10) inhibition of *Selenastrum* growth occurred at between 0.002 and 0.005 mg l^{-1} mercury for cell number (Figure 3.3 A) and chlorophyll *a* (Figure 3.3 C and D) measurements. No toxicity was observed when the growth rate was measured in terms of dry weight (Figure 3.3 B).

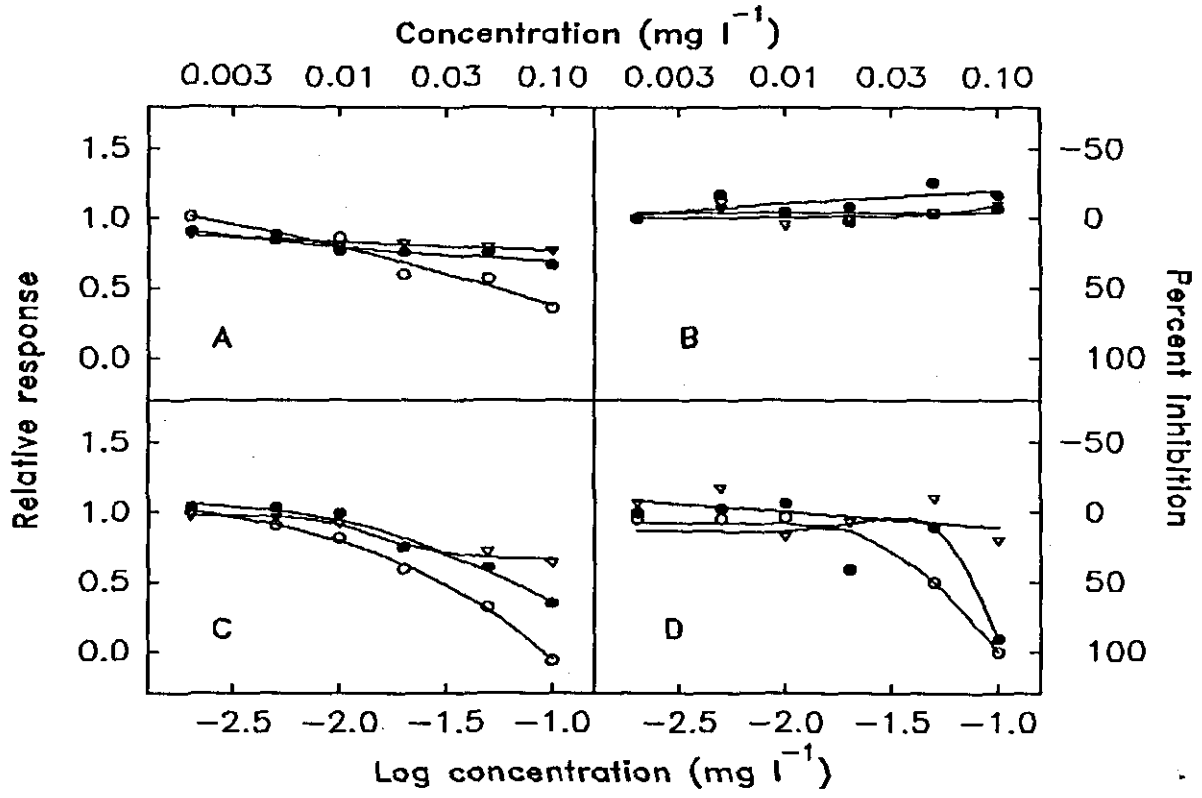


Fig. 3.3. The relative growth response of *Selenastrum capricornutum* to different concentrations of mercury. Relative responses were obtained from changes in cell numbers (n ml-1) (A), dry weight (mg l-1) (B), chlorophyll *a* content (µg l-1), measured fluorometrically (C) and spectrophotometrically (D).
(○ = 24h, ● = 48h and ▽= 72h)

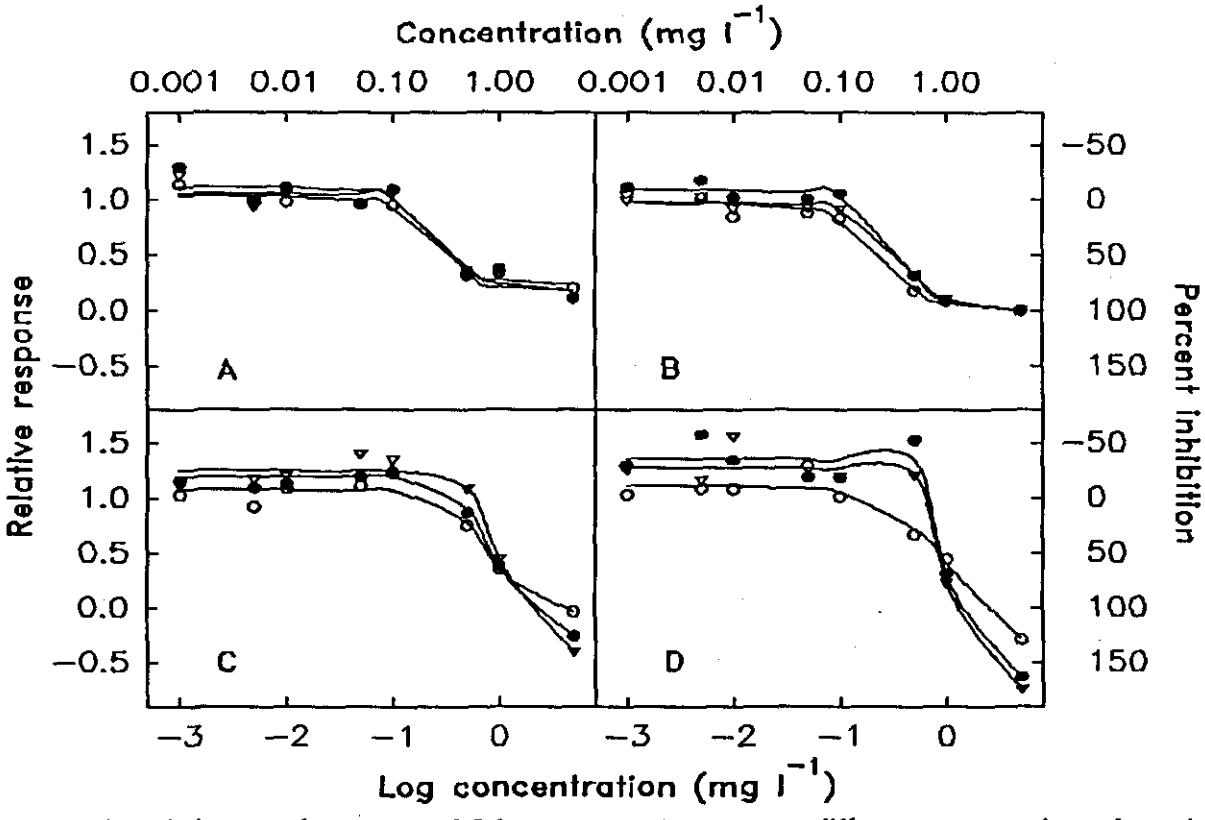


Fig. 3.4. The relative growth response of *Selenastrum capricornutum* to different concentrations of atrazine. Relative responses were obtained from changes in cell numbers (n ml-1) (A), dry weight (mg l-1) (B), chlorophyll *a* content (µg l-1), measured fluorometrically (C) and spectrophotometrically (D).
(○ = 24h, ● = 48h and ▽ = 72h)

The responses of *Selenastrum* to various concentrations of atrazine are shown in Figure 3.4. The results show that atrazine at low concentrations (0.001 - 0.1 mg l^{-1}) stimulated algal growth as measured in terms of the chlorophyll *a* content (Figure 3.4 C and D). The inhibition increased with an increase in the atrazine concentration of between 0.1 and 5 mg l^{-1}, a range of almost 0 to almost 100 % inhibition. It is also clear, from Figures 3.4 C and D, that below 1 mg l^{-1} *Selenastrum* was less inhibited by atrazine over time and it seems as though the algae recovered after an initial inhibition. This also applied to growth as measured in terms of cell numbers and dry weight (Figures 3.4 A and B).

Dose-response curves for *Selenastrum* exposed to different gusathion concentrations are shown in Figure 3.5. It is clear from the results that growth was not affected by gusathion over the concentration range of 1 x 10^{-5} to 1 mg l^{-1}.

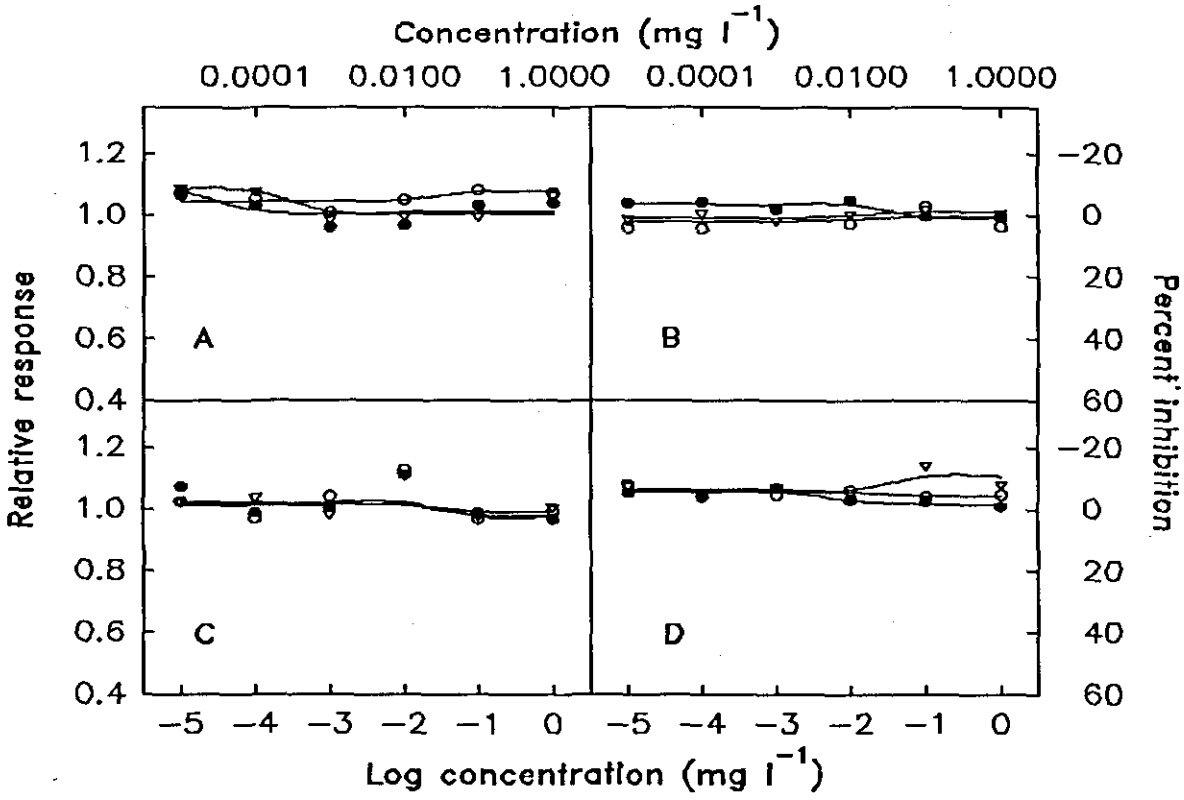


Fig. 3.5. The relative growth response of *Selenastrum capricornutum* to different concentrations of gusathion. Relative responses were obtained from changes in cell numbers (n ml-1) (A), dry weight (mg l-1) (B), chlorophyll *a* content (µg l-1), measured fluorometrically (C) and spectrophotometrically (D).
(○ = 24h, ● = 48h and ▽ = 72h)

Figure 3.6 shows the effect which phenol had on algal growth. Although no EC50 values were obtained in these experiments there exists some variance amongst the different parameters used for quantifying algal growth. With measurement of cell numbers (Figure 3.6 A) a steady decline in response was observed with an increase in the concentration of the phenol. On the other hand no observable effect (less than 10% inhibition) could be seen in terms of dry weight (Figure 3.6 B). An initial stimulation of growth by 15 % was seen at low concentrations in terms of fluorometric chlorophyll *a* measurements (Figure 3.6 C), after which growth was increasingly inhibited at higher phenol concentrations. Growth in terms of spectrophotometric chlorophyll *a* measurements (Figure 3.6 D) showed a time dependence where after 24 h exposure no effect was observed, whilst a 40 % inhibition was seen after 72 h exposure to 1 mg phenol l^{-1}.

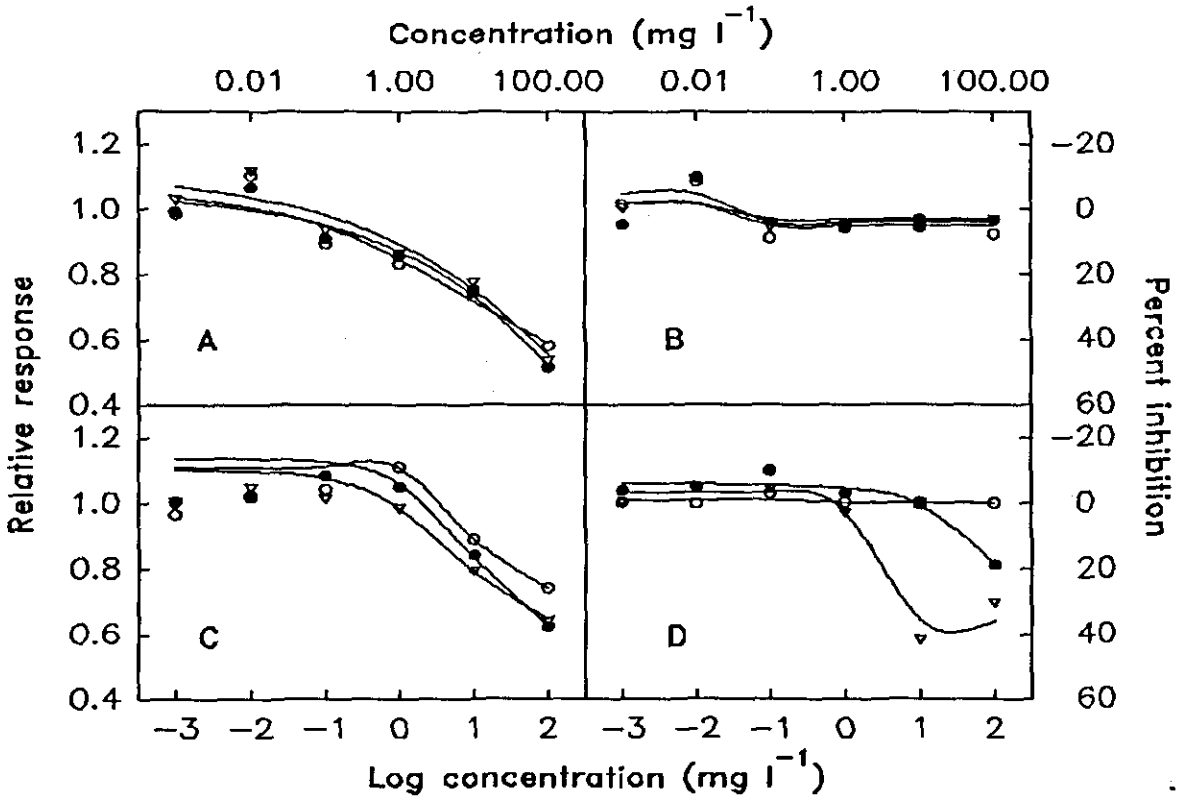


Fig. 3.6. The relative growth response of *Selenastrum capricornutum* to different concentrations of phenol. Relative responses were obtained from changes in cell numbers (n ml^{-1}) (A), dry weight (mg l^{-1}) (B), chlorophyll *a* content (µg l^{-1}), measured fluorometrically (C) and spectrophotometrically (D).
(○ = 24h, ● = 48h and ▽ = 72h)

Discussion

Toxicity tests using algae as test organisms provide an important method of assessing the biological effects of elements in solution. Since algae are at the base of most aquatic food webs, any factor affecting them will have consequences for entire systems. The results presented, clearly show that algae (*Selenastrum*) are sensitive to certain toxic substances and that the degree of toxicity depends on the particular substance, as well as the growth parameters used. The EC values are given in Table 3.1 for the various compounds tested.

Christensen and Nyholm (1984) reported EC values of 0.0263, 0.0485 and 0.0717 mg l^{-1} for copper (EC10, EC50 and EC90 respectively) which is one order of magnitude lower than the values of 0.151, 0.227 and 0.290 mg l^{-1} as determined in this study in terms of fluorometric chlorophyll *a* measurements after 24 h of exposure (Table 3.1). According to Wong (1989) both *Selenastrum* and *Scenedesmus* were unable to survive a Cu concentration above 90 µg l^{-1} which is in the same order as the EC90 value reported by Christensen and Nyholm (1984). Our results suggest that the degree of copper toxicity decreases with time as indicated by all four growth parameters. This indicates an adaptation to copper. The differences were highly significant ($P = 0.009$) between 24 and 48 h exposure and significant ($P = 0.05$) between 48 and 72 h exposure. It is, therefore, imperative that the duration of toxicity tests of copper, employing algae, be as short as possible to ensure maximum sensitivity.

Table 3.1. Summary of the results from algal growth potential toxicity tests. EC-values (in mg l^{-1}) were calculated from dose-response curves. Growth rates were determined from measurements of dry weight, cell numbers, chlorophyll *a* concentrations determined spectrophotometrically and fluorometrically. (nr = no response)

Compound	Time	Cell numbers			Dry weight			Chlorophyll *a*					
								Spectrophotometric			Fluorometric		
		EC10	EC50	EC90	EC10	EC50	EC90	EC10	EC50	EC90	EC10	EC50	EC90
Copper	24h	0.160	0.207	nr	nr	0.699	1.239	0.254	0.312	0.349	0.151	0.227	0.290
	48h	0.427	0.505	nr	0.603	0.940	nr	0.263	0.311	0.336	0.409	0.450	0.475
	72h	0.497	0.688	1.146	0.680	0.917	0.960	0.340	0.392	0.407	0.431	0.499	0.524
Cadmium	24h	0.092	0.104	nr	0.494	0.713	nr	0.116	0.161	0.200	0.093	0.139	0.173
	48h	0.093	0.109	nr	0.422	0.479	nr	0.039	0.126	0.199	0.063	0.111	0.154
	72h	0.093	0.164	nr	0.342	0.452	nr	0.033	0.097	0.135	0.053	0.101	0.136
Mercury	24h	0.005	0.053	nr	nr	nr	nr	0.002	0.050	0.052	0.005	0.030	0.076
	48h	0.002	nr	nr	nr	nr	nr	0.047	0.094	0.100	0.013	0.063	nr
	72h	0.001	nr	nr	nr	nr	nr	0.094	nr	nr	0.011	nr	nr
Atrazine	24h	0.110	0.340	nr	2.802	nr	nr	0.279	0.838	1.847	0.375	0.784	1.715
	48h	0.148	0.377	nr	nr	nr	nr	0.948	0.985	1.018	0.500	0.856	1.409
	72h	0.167	0.359	nr	nr	nr	nr	0.743	0.902	1.053	0.670	0.960	1.322
Gusathion	24h	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
	48h	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
	72h	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
Phenol	24h	0.329	nr	nr	nr	nr	nr	nr	nr	nr	9.576	nr	nr
	48h	0.495	nr	nr	nr	nr	nr	42.55	nr	nr	5.946	nr	nr
	72h	0.969	nr	nr	nr	nr	nr	1.199	nr	nr	3.003	nr	nr

This investigation confirms reports that cadmium is extremely toxic to freshwater algae. An EC50 value for cadmium of 0.019 mg l^{-1} was reported by Vocke *et al.* (1980) which is five times lower than the EC50 value of ± 0.1 mg Cd l^{-1} as determined in our investigation for *Selenastrum* growth in terms of chlorophyll *a* measurements (Table 3.1). Vocke *et al.* (1980) used a two week exposure test, whilst ours was at maximum only after 72 h. It may well be that they could detect Cd concentrations as low as 0.019 mg l^{-1}, but it is also possible that some or other factor became limiting in their assays. This caution is confirmed by Nyholm (1985) who pointed out that tests should be conducted over an exposure period of only 2 to 3 days to avoid the possible effect that depletion of nutrients might have on the end result.

Harriss *et al.* (1970), noted that the toxicity of mercurial compounds decreased with increasing cell concentrations. Our results show that algal growth was inhibited over time by mercury, as indicated by a decrease in cell numbers with time as compared to the control culture (Figure 3.3 A). This increase in cell numbers could possibly explain why EC50 and EC90 values could not be calculated for mercury after 72 h of exposure and why the EC10 values increased with time. The EC50 value for mercury, as derived from chlorophyll *a* fluorescence measurements, of 0.030 mg l^{-1} (Table 3.1) compares well with the 0.033 mg l^{-1} as reported by Vocke *et al.* (1980).

Atrazine generally inhibited algal biomass growth (measured as cell numbers, dry weight, and chlorophyll *a* content determined spectrophotometrically or fluorometrically). Growth occurred over all three days but the growth rate decreased over time as can be seen from EC values in Table 3.1. The lowest EC50 value (0.340 mg l^{-1}) was obtained from calculations with cell numbers. The EC50 values obtained with cell numbers and chlorophyll *a* determined spectrophotometrically increase between day 1 and 2 and decrease between days 2 and 3. Fluorometric chlorophyll a measurements shows a progressive increase in EC10 and 50 values from day 1 to 3. The significance of the results obtained with atrazine is that although the EC10 and EC50 values fluctuate over time and amongst measurements the lowest values were obtained after 24 h growth. EC90 values, where it could be calculated (only chlorophyll a measurements), shows the opposite with the lowest value after 72 h growth. It can thus be concluded that the effect of atrazine decreased over time up to a certain level (EC90) which can then be considered as the "lethal" dose after which recovery was no longer possible. The algae starts recovering from day 2, which is consistent with findings of researchers who exposed isolated algal species to atrazine in the laboratory (Walsh, 1972; Abou-Waly *et al.*, 1991a). Although Abou-Waly *et al.* (1991a) did not calculate EC values from their chlorophyll a measurements, they reported concentrations of 0.23 to 0.42 mg l^{-1} atrazine, which significantly reduced *Selenastrum capricornutum* growth. This agrees favourably with our EC10 values (i.e. first observable effect) of 0.28 to 0.38 mg l^{-1} calculated from chlorophyll *a* measurements after 24 h (Table 3.1).

Although EC90 values could not be caculated, results obtained from cell number measurements were the most sensitive and EC10 and 50 values were determined. The dry weight measurements fails to give any meaningful results (Table 3.1) and this method of determining algal growth should be considered inferior, although more sensitive than chlorophyll *a* measurements.

Gusathion does not show any effect on algal growth as determined from our experiments. Mohapatra and Mohanty (1992) support this when they found that dimethoate or 0, 0-dimethyl-S(-N-methyl-carbamoylethyl)-dithiophosphate (an organophosphorus pesticide) at 10 and 25 mg l^{-1} did not show any change in the sigmoidal pattern of growth of *Chlorella vulgaris* (a green alga) grown for 10 d on nutrient agar plates where cell multiplication was observed with a microscope. At a concentration of 1 mg l^{-1} no significant change in the growth pattern was observed by them. This concentration is also the highest concentration of gusathion that *Selenastrum* was exposed to in our study with similar results. However, they reported sublethal concentrations (LC50s which is equal to EC50) of 28.5 mg l^{-1}. This does not mean that *Selenastrum* would have responded in the same way if we would have tested higher concentrations of gusathion because Kühn and Pattard (1990) reported EC50 values ranging from 1.1 mg l^{-1} to 2.8 mg l^{-1} with a 72 h test period where *Scenedesmus*

subspicatus (batch cultures in Erlenmeyer flasks) growth rates were calculated when exposed to another organophosphorus compound namely; phosphoric acid tributyl ester. All this means that although organophospates do have an influence on algal growth it varies amongst specific substances and amongst test species and test methods.

Phenol volatility could have a marked influence on the results obtained from toxicity measurements. This effect can be offset, however, by providing appropriate modifications to better seal individual flasks and improving experimental design. Thellen *et al.* (1989) reported an EC50 value of 69.7 mg l^{-1} for phenol obtained with a microplate toxicity assay over 96 h. None of the results obtained in this study was close to this value excepting for an EC10 of 42.55 mg l^{-1} obtained after 48 h exposure in terms of chlorophyll *a* measured spectrophotometrically (Table 3.1). No EC50 values were obtained for phenol in the concentration range tested, which emphasises the fact that when working with a volatile substance care should be taken to improve the experimental design. Our experiments did not allow for losses of volatile substances.

Because of the negative values of the growth rate, especially at high concentrations of the tested toxic compounds, after one day's exposure (Figures 3.1, 3.2 and 3.4) and the ability of the algae to recover with time, results from such tests should be interpreted with caution. We are of the opinion that more meaningful results will be obtained when measurements are made every day for at least 3 days after exposure. A further complication is that it is difficult to determine whether individual algal cells are actually dead, and which treatments are reversible or not.

Comparing the four different growth parameters the following can be seen (See Table 3.1); i) dry weight measurements were the least sensitive towards the test compounds, ii) the highest sensitivity for copper was obtained after 24 h with fluorometric chlorophyll *a* measurements, iii) cadmium had an increasing effect over time as measured by all the parameters except cell numbers with the highest sensitivity obtained after 72 h from spectrophotometric chlorophyll *a* measurements, iv) mercury had a decreasing effect over time but again cell numbers showed the opposite and the highest sensitivity was obtained with spectrophotometric chlorophyll *a* measurements after 24 h, v) cell numbers were the most sensitive parameter for atrazine after 24 h which was the same with phenol.

Cell numbers fail to distinguish between living and dead cells and also gave contradictory results as far as cadmium and mercury were concerned which renders it an inappropriate parameter for the purpose of this experiment. There was no big difference between the two different chlorophyll *a* parameters although fluorometric measurements gave the higher sensitivity three out of five times (i.e. copper, atrazine and phenol). The fluorometric chlorophyll *a* measurements are therefore considered to give the better overall estimate of algal growth potential under toxicity stress and these results were used for further comparisons with the other bioassays (See Chapter 8).

CHAPTER 4

THE USE OF ALGAL *IN VIVO* FLUORESCENCE AS AN INDICATOR OF TOXICITY

Introduction

Ecologists have struggled for decades to understand how biological, chemical and physical processes regulate the abundance, distribution and productivity of aquatic organisms (Parsons *et al.*, 1984). Progress in understanding phytoplankton dynamics is to a large extent due to two techniques, namely the ^{14}C - method for measuring primary productivity (Steemann Nielsen, 1952) and the fluorometric method of measuring chlorophyll (Lorenzen, 1966).

The characteristic wavelengths of light absorption and fluorescence of the various chl-protein complexes is influenced markedly by the protein moiety. The pigment-protein complexes are organized in membranes which provide a means by which gradients in electrochemical energy can be produced and maintained (Figure 4.1). Photosynthesis can be viewed as the controlled production and dissipation of an electrochemical gradient where the oxidation of water provides a source of electrons and the initial driving force is the free energy released by de-excitation of an excited pigment molecule.

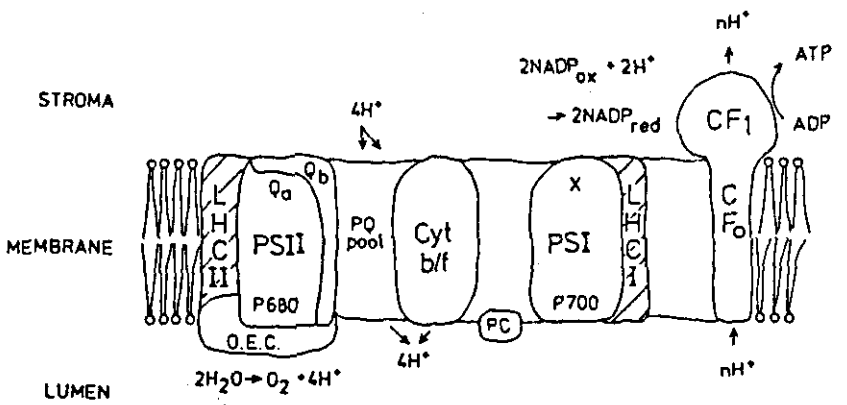


Fig. 4.1. Diagram indicating how non-cyclic electron transfer is thought to be coupled to proton movements across the thylakoid membrane. (a) The oxidation of water takes place in the lumen, leading to the release of protons, whilst proton uptake occurs in the stroma when NADP is reduced. (b) The reduction of quinones, which requires protons as well as electrons, takes place at the stromal side of the membrane, whilst oxidation of plastoquinone and proton release occurs on the lumen side, leading to proton translocation. From Hipkins, 1986.

The *in vivo* fluorescence yield is produced primarily by two processes. Excitation energy will be lost as heat, and it will also find its way to special chlorophyll molecules, the reaction centres, where it is used to remove an electron from the orbital system and drive it to a more electrically negative system. In all photosynthetic algae there are two types of reaction centres, namely photosystem I (PSI) and photosystem II (PSII). The absorption change accompanying the oxidation of PSI is maximal at 700 nm and hence the pigment is often called P700. Similarly, the absorption change accompanying the oxidation of PSII is maximal at 680 nm and the pigment is often called P680. Excitation energy is delivered to the reaction centres by the antenna chlorophyll molecules, organized in proteins near the reaction centres.

The fluorescence yield from P680 and its antenna is very large so that almost all of the chlorophyll fluorescence emanating from an algal cell is associated with photosystem II. It should be pointed out that there are many pigments in algal cells which are capable of *in vitro* fluorescing. Amongst these are chlorophyll *b*, chlorophylls *c*1 and *c*2, phycoerythrin,

phycocyanin, and allophycocyanin. *In vivo* these pigments normally transfer virtually all the excitation energy they receive to chlorophyll *a* and do not fluoresce (Falkowski and Kiefer, 1985).

In 1963, Duysens and Sweers hypothesized the existence of a fluorescence quencher, called Q, which is associated with PSII. When a photon is absorbed by antenna pigments and transferred to PSII, it can drive an electron to Q. If Q is oxidized the PSII "trap" is said to be "open". Under these conditions the electron can reduce Q to Q^- and excitation energy is converted to vibrational energy (heat), or transferred to another trap. From both biophysical and biochemical evidence, it is believed that Q is a quinone tightly bound to a protein. Q transfers electrons to a secondary quinone called "Q_B". Q_B loosely binds to or near Q and can also dissociate after it receives a pair of electrons and protons.

Many herbicides, including 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and atrazine compete with Q_B for a binding site on Q. In the presence of such an inhibitor the complex Q-I is formed and PSII traps cannot turn over more than once. Thus, even in the presence of light, electron flow from water to Q_B (and subsequently PSI and CO_2) is blocked. Under such conditions fluorescence rises to a maximum value which will be referred to as F_m i.e. maximum fluorescence, (Falkowski and Kiefer, 1985).

Fluorescence is minimal, F_o, when all reaction centres are open, and maximal, F_m, when all the reaction centres are closed. The fraction of reaction centres that are open at any time can be obtained from the fluorescence values F, F_o and F_m. An index of toxicity, i.e. the extend of Q_A reduction and the overall inhibition of electron flow by (toxic) compounds, can be obtained from a measure of these three fluorescence values.

The aim of this study was, therefore, to test whether or not *in vivo* fluorescence of algae could be used as a fast and effective screening bioassay to detect waterborne toxic substances. Experiments were conducted with selected substances (See Chapter 2, Table 2.1) and a sample of tertiary treated municipal sewage effluent with unknown substance.

Material and methods

Test organisms and growth conditions - See Chapter 2, Paragraph 2.1.

Measurement of in vivo *fluorescence* - All fluorescence measurements were made on a Hitachi Model F-2000 Fluorescence Spectrophotometer, Hitachi Ltd., Tokyo, Japan. The light source was a 150 W Xenon short arc lamp with ozone self-dissociation function. The two monochromators as used on the excitation and emission sides are large stigmatic concave gratings having 900 lines mm^{-1}.

In this study 14 individual *in vivo* fluorescence emission spectra were obtained for *Selenastrum* and *Chlorella* at excitation wavelengths ranging from 360 nm to 620 nm. The 14 emission spectra were plotted sequentially to form an isometric projection of fluorescence over a range of excitation and emission wavelengths which is commonly referred to as an excitation-emission-matrix (EEM) (Zung *et al.*, 1990). From the EEMs the optimal excitation and emission wavelengths for both algae were determined. These predetermined excitation and emission wavelengths were set with a 10 nm bandpass prior to the measurements. Integration time was 0.5 sec and the maximum time taken for an excitation or emission scan was ± 1.5 min.

Fifty ml aliquots of the algal culture were taken for each measurement. These aliquots were kept in the dark for 20 min to allow the cells to adapt to the dark. The compounds listed in Table 2.2, Chapter 2, were added to the algae after dark adaptation at different concentrations and the fluorescence intensities were measured immediately (time zero). Sterile distilled water was added as control and DCMU (10^{-3} M) was added to obtain maximum fluorescence (F_m). Fluorescence measurements were repeated after 30 min, 1 h and 4 h after exposure to the treatments. Each measurement was done in triplicate.

Calculations - Results were expressed as an index of toxicity relative to control. The index for photosynthetic capacity, F_t (which can also be seen as an index of toxicity of a pollutant) was calculated as;

$$F_t = (F_m - F) / (F_m - F_o)$$

where F_m = maximum fluorescence (fluorescence with DCMU added - closed state), F_o = natural fluorescence (control fluorescence - open state or the dark adapted state), F = the measured fluorescence of a sample and F_t = the index of toxicity (fraction of reaction centres open under specific conditions) (Falkowski and Kiefer, 1985). This equation is basic to an understanding of the relationship between variable fluorescence and the rate of electron transport or rate of photosynthesis. With the index of toxicity (F_t) taken as the measured response (r) [See Equation (1), Chapter 2], the data was further dealt with as described in Chapter 2, so as to construct dose-response curves and determine EC values.

Results

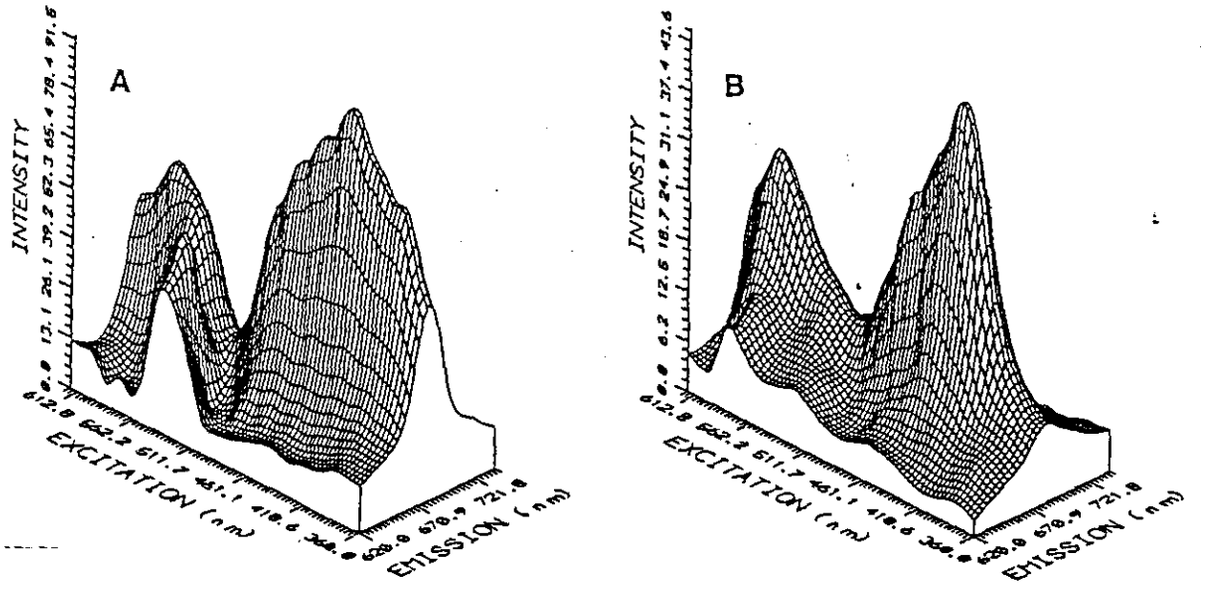


Fig. 4.2. EEM's of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B).

Representative EEM spectra of *Selenastrum* and *Chlorella* are shown in Figures 4.2 A and B. The EEMs of *Selenastrum* and *Chlorella* reveal only *in vivo* chlorophyll *a* fluorescence with emission for both at 680 nm. The spectral detail reveals that excitation absorption occurs at two wavelength regions i.e. at 430 nm and 590 nm. The differences in fluorescence intensity could be attributed to a difference in *in vivo* chlorophyll *a* contents and different concentrations of cells (biomass) used for the two species in constructing the EEM's.

No effects (less than 10 % inhibition which is the same as a relative response of 0.9, See Chapter 2, Paragraph 2.2) were observed when algae were exposed to heavy metals; copper, cadmium and mercury (Figures 4.3, 4.4 and 4.5). Shown in Figure 4.3 A and B is the effect which copper had on the fluorescence yield of intact *Selenastrum* and *Chlorella* cells. At low concentrations an initial stimulation of between 0 and 4 % (relative response of between 1 and 1.04) was observed. After 4 h exposure to a copper concentration of 2 mg l^{-1} this initial stimulation was inhibited to a relative response of 0.925 or 7.5 % inhibition. Adding the 4 % stimulation one could argue that 11.5 % inhibition did occur after 4 h exposure to 2 mg l^{-1} copper. However, it was not possible to calculate an EC90 value (i.e. 10 % inhibition or 0.9 relative response) from the results obtained in the concentration range used for both test organisms. Although an initial stimulation was seen at low concentrations, it varied over time and between the two species used and no conclusion could be made. The same applies to the higher concentrations where a tendency was seen

towards a declining relative response, but again with variations over time and between species.

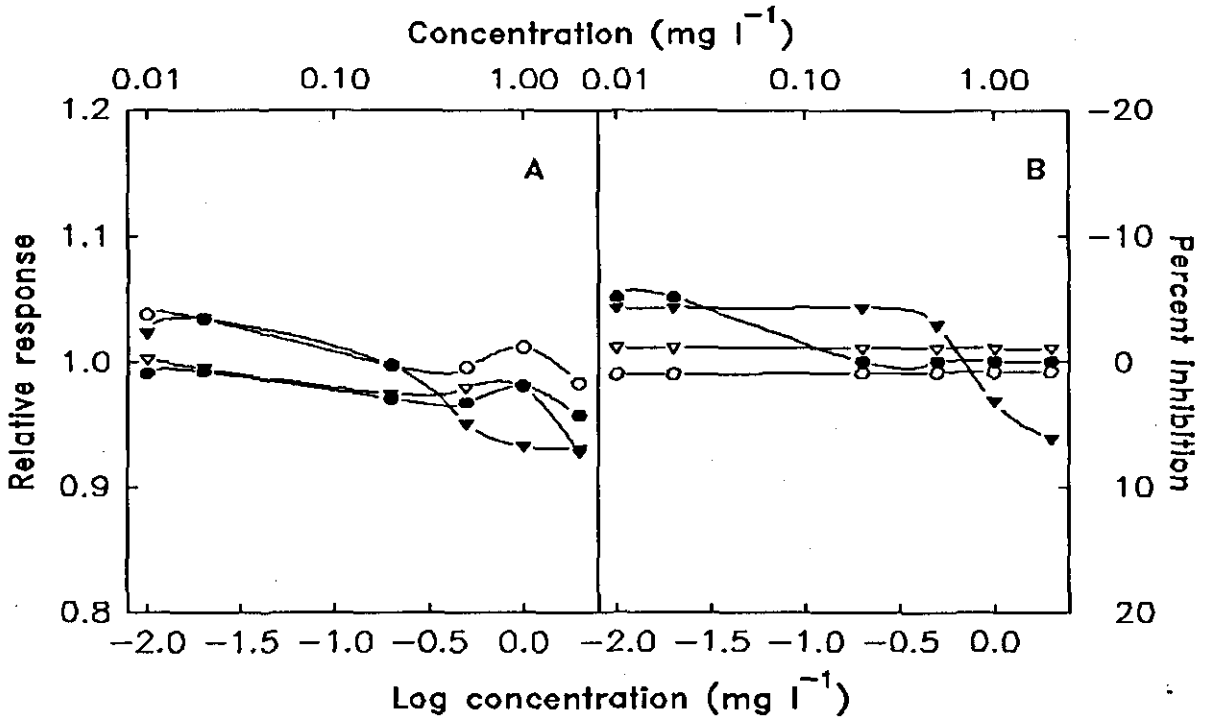


Fig. 4.3. Copper toxicity of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B). Measurements were taken after 0 h (○), 30 min (●), 1 h (▽) and 4 h (▼).

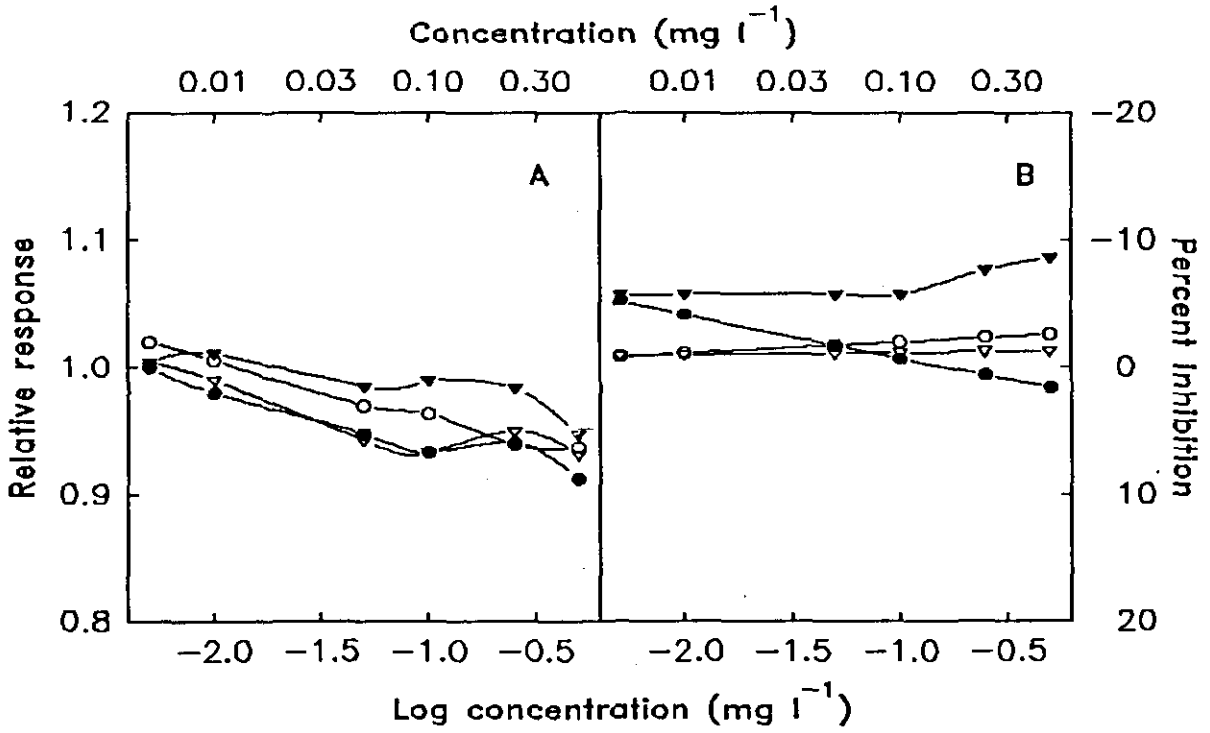


Fig. 4.4. Cadmium toxicity of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B). Measurements were taken after 0 h (○), 30 min (●), 1 h (▽) and 4 h (▼).

Figure 4.4 A and B show the effect of cadmium on *in vivo* chlorophyll fluorescence of *Selenastrum* and *Chlorella*. The response measured with *Selenastrum* (Figure 4.4 A) shows no initial stimulation and an inhibition of 6 % after 30 min exposure to cadmium at a concentration of 5 mg l^{-1}. This was the highest inhibition observed because after 1 h exposure to the same cadmium concentration the inhibition decreased to 4 % and after 4 h to 2.5 %. *Chlorella* (Figure 4.4 B) shows a somewhat different response. After 30 min and 4 h exposure to 0.005 mg l^{-1} cadmium an initial stimulative response of 5 % was observed. The initial response and the response after 1 h exposure remained the same for all the cadmium concentrations tested. Increasing cadmium concentration fivefold, from 0.1 to 0.5 mg l^{-1}, resulted in a further stimulation to 8 % in the sample exposed for 4 h.

The effect of mercury on *in vivo* chlorophyll fluorescence is illustrated in Figure 4.5. *Selenastrum* (Figure 4.5 A) shows a slight inhibition with an increase in concentration. However, with the mercury concentration ranging from 0.005 to 0.2 mg l^{-1} i.e a 40 times increase the response ranged from 1 to 0.95 i.e only 5 % inhibition which may be considered as no observable effect (less than 10 % inhibition). *Chlorella* (Figure 4.5 B) displayed a similar response and the only difference is that a stimulation after 30 min and after 4 h exposure of 5 % is seen which decrease to zero at high concentrations.

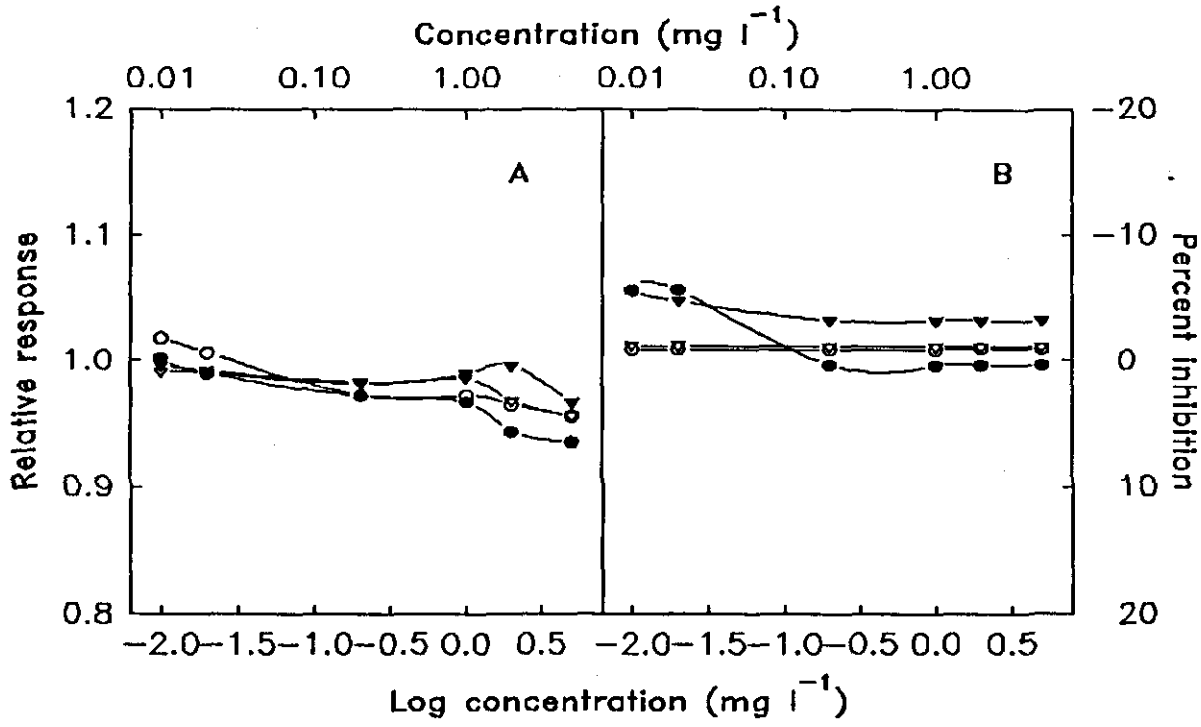


Fig. 4.5. Mercury toxicity of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B). Measurements were taken after 0 h (○), 30 min (●), 1 h (▽) and 4 h (▼).

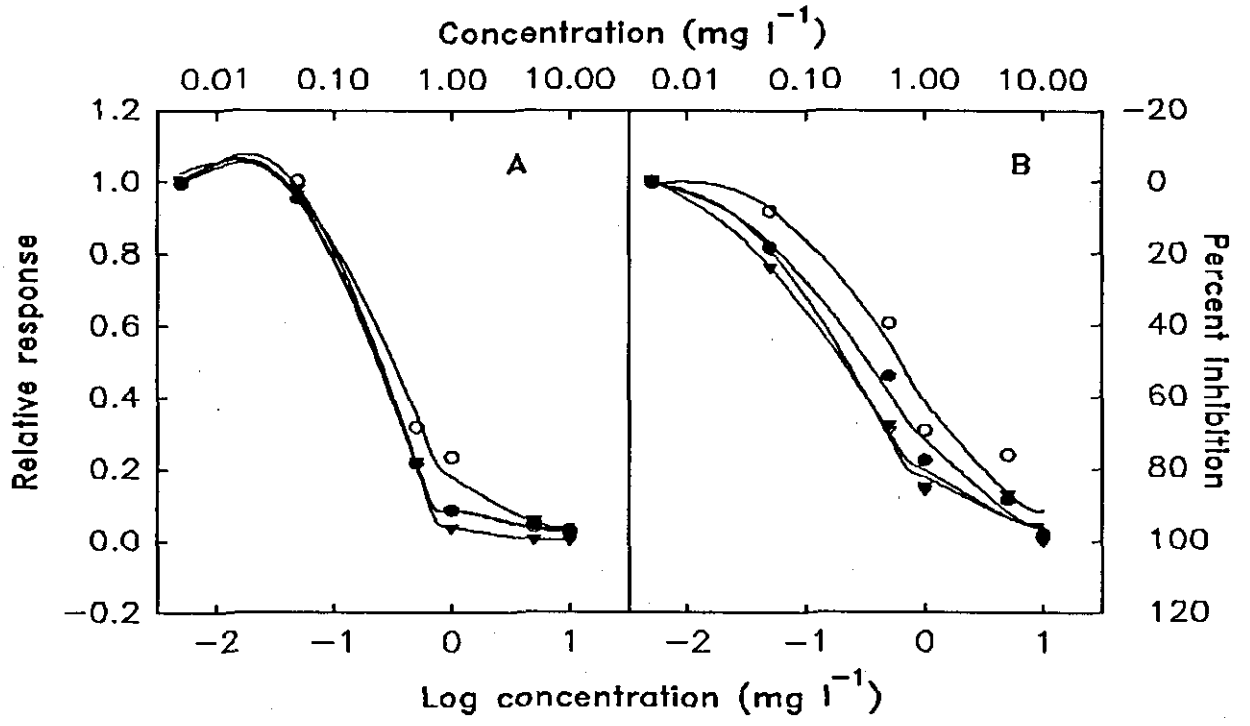


Fig. 4.6. Atrazine toxicity of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B). Measurements were taken after 0 h (○), 30 min (●), 1 h (▽) and 4 h (▼).

Shown in Figure 4.6 is the effect of atrazine on *in vivo* chlorophyll *a* fluorescence of *Selenastrum* (Figure 4.6 A) and *Chlorella* (Figure 4.6 B). At a concentration of 0.005 mg l^{-1} no inhibition was observed for both algae. A ten times increase in atrazine concentration resulted in a slight differentiation of the inhibition over time for both algae. *Selenastrum* indicated after 4 h exposure a 5 % inhibition whereas *Chlorella* showed an immediate 5 % inhibition (0.95 relative response) when exposed to 0.05 mg l^{-1} and after 4 h exposure a 25 % inhibition. Thus *Chlorella* was four times more sensitive than *Selenastrum*. A concentration of 0.5 mg l^{-1} atrazine inhibits 70 % of the energy transfer between PSII and PSI in *Selenastrum* immediately and almost 80 % after 30 min and up to 4 h exposure. The extent of inhibition in *Chlorella* was 40 % immediately, 55 % after 30 min and 70 % after 1 h and after 4 h exposure. The inhibition of *Selenastrum* increased to 80 % when exposed to 1 mg l^{-1} measured just after dosage and to 90 % after 30 min and 95 % after 1 and 4 h exposure. *Chlorella* at this same concentration showed an inhibition of 70 %, 78 % and 85 % respectively. At higher atrazine concentrations 100 % inhibition was found for *Selenastrum*. An increase of five times in concentration to 5 mg l^{-1} only inhibited the *in vivo* chlorophyll fluorescence of *Chlorella* a further 5 % and 10 mg l^{-1} gave a 100 % inhibition.

Figure 4.7 shows the effect of gusathion on chlorophyll *a* fluorescence. From the results it is clear that gusathion had no observable effect (10 % inhibition) on *in vivo* chlorophyll *a* fluorescence of both *Selenastrum* (Figure 4.7 A) and *Chlorella* (Figure 4.7 B). What is significant about the results obtained with *Chlorella*, however, was a stimulation of 5 % at high concentration (1 mg l^{-1}) dosages.

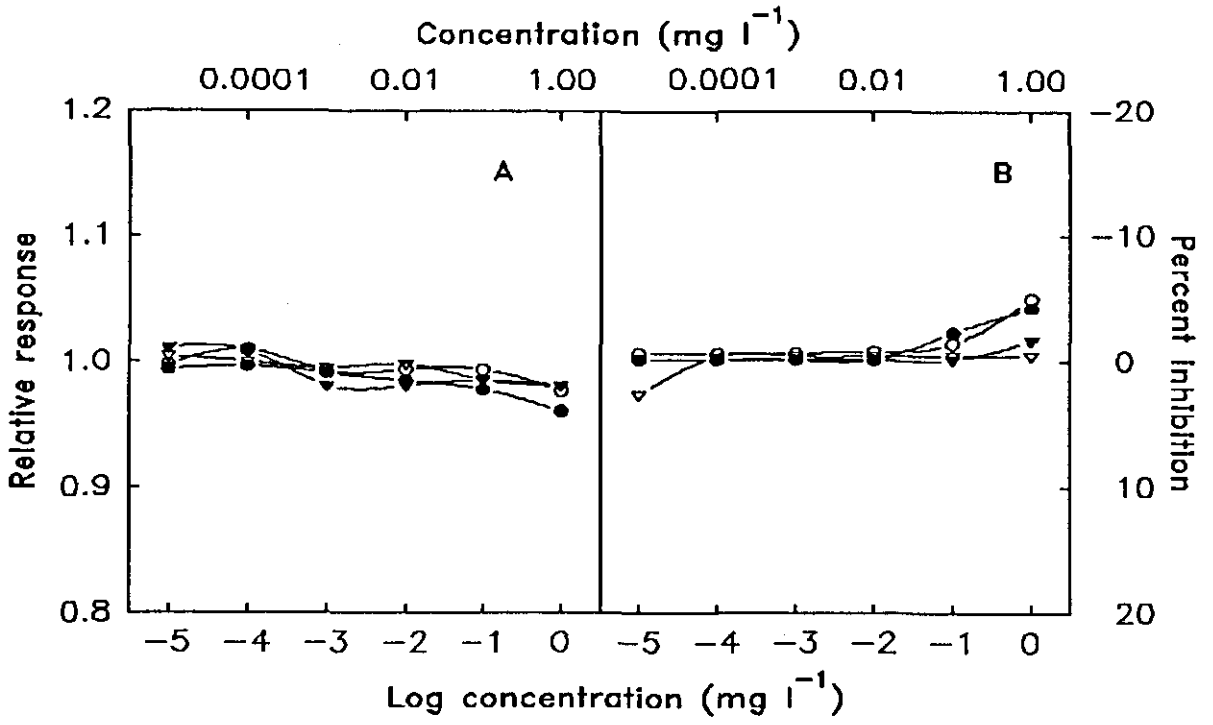


Fig. 4.7. Gusathion toxicity of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B). Measurements were taken after 0 h (○), 30 min (●), 1 h (▽) and 4 h (▼).

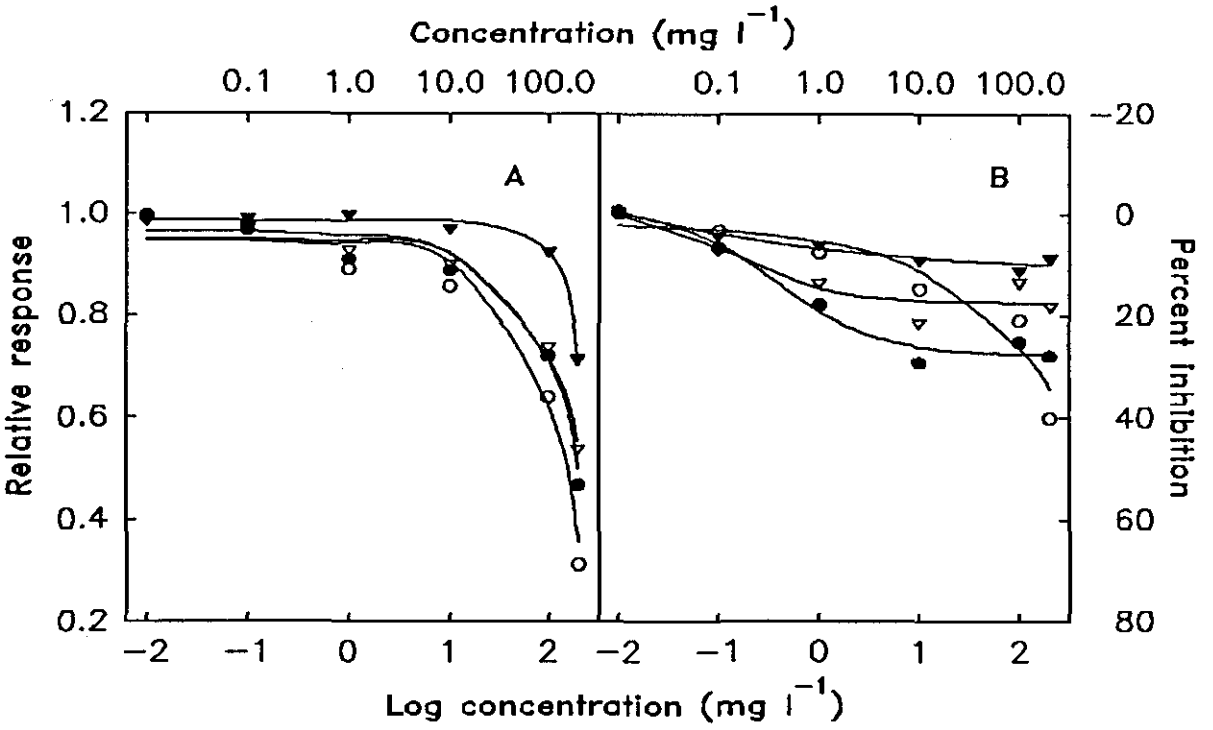


Fig. 4.8. Phenol toxicity of *Selenastrum capricornutum* (A) and *Chlorella vulgaris* (B). Measurements were taken after 0 h (○), 30 min (●), 1 h (▽) and 4 h (▼).

The effect which phenol had on *in vivo* chlorophyll *a* fluorescence is shown in Figure 4.8. From Figure 4.8 A it can be seen that *Selenastrum* is tolerant towards phenol up to a concentration of 0.1 mg l^{-1} whereafter there is no differentiation in response (1 %

inhibition) over time. With an increase to 1 mg l^{-1} the inhibition increased to almost 10 % within seconds (zero time exposure) whereafter it decreased by about 5 % after an hours exposure. When the exposure at this same concentration was extended to 4 h almost no inhibition (1 %) was observed. An increase of ten times in the concentration resulted in a further 5 % increase in the response at zero time. When the concentration was increased to 100 mg l^{-1} the inhibition increased to 35 % initially (zero time exposure), 25 % after 30 min and after 1 h, and 5 % after 4 h exposure. At 200 mg l^{-1} it was 70, 52, 48 and 30 %, respectively. *Chlorella* appeared to be more sensitive to phenol. At 0.1 mg l^{-1} the inhibition was almost 8 % after 30 min and after 1 h, 4 % after 4 h exposure and 3 % at zero time. With 1 mg l^{-1} the situation changed to 18 % inhibition after 30 min, 15 % after 1 h, 7 % after 0 h and 5 % after 4 h and 10 mg l^{-1} gave 30, 20, 15 and 10 % inhibition, respectively. It can be seen that the response increased progressively after zero time so much that a twenty times increase in the concentration of 10 mg l^{-1} to 200 mg l^{-1} resulted in an inhibition increment of 25 %.

Tertiary treated municipal sewage effluent at concentrations of 25, 50, 75 and 100 % had no effect on *in vivo* chlorophyll *a* fluorescence of *Selenastrum* or *Chlorella*.

Table 4.1. Summary of the results from *in vivo* fluorescence bioassays with *Selenastrum capricornutum* and *Chlorella vulgaris*. EC values are given in mg l^{-1}. *nr = no response.

Compound	Exposure time	*Selenastrum*			*Chlorella*		
		EC10	EC50	EC90	EC10	EC50	EC90
Atrazine	0 h	0.146	0.335	1.900	0.070	0.625	7.679
	0.5 h	0.099	0.246	0.993	0.027	0.325	4.005
	1 h	0.093	0.253	0.901	0.029	0.201	2.269
	4 h	0.079	0.232	0.697	0.020	0.194	2.712
Phenol	0 h	11.22	145.1	*nr	6.955	nr	nr
	0.5 h	19.73	nr	nr	0.209	nr	nr
	1 h	16.47	nr	nr	0.219	nr	nr
	4 h	115.0	nr	nr	172.3	nr	nr

Discussion

From the EEMs (Figure 4.2) excitation and emission wavelengths were derived from which the *in vivo* chlorophyll *a* fluorescence inhibitory effects of toxicants were evaluated. Zung *et al.* (1990) reported an emission wavelength of 667 nm and an excitation wavelength of 471 nm for *Chlorella vulgaris* as being typical of chlorophyll *a* fluorescence. The wavelengths 430 nm and 680 nm were derived from this this study for *Selenastrum* and *Chlorella* excitation and emission, respectively. This agrees, in broad terms, with previously reported results and was used in all our experiments.

Amongst all the substances tested the herbicide atrazine (Figure 4.6) and phenol (Figure 4.8) were the only two to have any observable effect (more than 10 % inhibition) on *in vivo* chlorophyll *a* fluorescence. In describing an apparatus for the continuous recording of algal fluorescence Benecke *et al.* (1982) recorded sensitivity values of 0.0325 mg l^{-1} atrazine, 0.005 mg l^{-1} $HgCl_2$, 0.003 mg l^{-1} $CuSO_4$ and 0.007 mg l^{-1} $Cd(NO_3)_2$. These values are much lower than EC10 values reported in Table 4.1 and standards in Table 2.2. However, it is not clear from their results what the degree of inhibition relative to the normal fluorescence was. Schmidt (1986), in using the method of Benecke *et al.* (1982), in a computerized bioassay, mentioned a 20 % deviation from the normal as an indication of toxicity. From this it is clear that the concentrations reported by Benecke *et al.* (1982) should be interpreted with caution.

In examining of the data (Table 4.1) closer, it can be seen that initially *Chlorella* did not respond as quickly as *Selenastrum* to the addition of atrazine. An EC50 value of 625 µg l^{-1} was recorded for *Chlorella* against the 335 µg l^{-1} for *Selenastrum*. After 4 h of exposure *Chlorella* was more susceptible to atrazine with an EC50 value of 194 µg l^{-1} as opposed to 232 µg l^{-1} for *Selenastrum*.

The EC10 values (Table 4.1) clearly illustrated the diminishing effect which phenol had on *Selenastrum* over time. *Chlorella* showed an initial inhibition at very low concentrations after 30 min and after 1 h opposed to immediate dosage at the same level (0.2 mg l^{-1} against 7 mg l^{-1}). After 4 h of exposure to phenol the effective concentration increased to 172 mg l^{-1} at EC10 level. This increase is attributed to the volatility of phenol (Galassi and Vighi, 1981) resulting in a decreased inhibition over time.

Mueller *et al.* (1992) published values where fluorescence spectroscopy was used to detect herbicides in various matrices. Although some herbicides were detected that do fluoresce it was at different excitation and emission wavelengths than that of chlorophyll *a*. Interferences with chlorophyll *a* fluorescence in our measurements could, therefore, be ruled out. Zung *et al.* (1990), however, warned that *in vivo* chlorophyll *a* excitation and emission wavelengths could vary between species in natural water and different environmental conditions. This could mean that with continuous *in situ* measurements of chlorophyll *a* fluorescence it is not always chlorophyll *a* alone that do fluoresce at specified wavelengths.

DCMU-fluorescence gives information about the "pool size" of active PSII reaction centres (Falkowski and Kiefer, 1985). It also reflects the photosynthetic ability of algae (Vincent, 1980) by expressing their potential for photochemistry. Cullen *et al.*, (1986) reported that it can be useful as a fast and simple method to indicate inhibition of photosynthesis in cases of contamination. The procedure of estimating photosynthetic capacity from $F_m - F_o$ poses some problems. In reality it is difficult to fully oxidize Q. The equilibrium constant for reaction $Q^- + e + Q_B <===> Q^-Q_B^-$ appears to be close to unity. Furthermore, Q_B^- cannot dissociate from Q until it receives a second electron. At a given time there is a mixture of Q, Q^-, Q_B, Q Q_B^- and $Q^-Q_B^-$ in the chloroplast. Q_B^- is also stable for a long time (many minutes) (Falkowski and Kiefer, 1985).

Care should be taken when measuring fluorescence induction so as to obtain the pattern for the redox reaction Q. Dark incubation before the measurement should, therefore, be longer than the lifetime of Q_B^- (>10 min) and is essential for the oxidation of P680. Ishimaru *et al.*, (1985) reported that the duration of the dark adaptation period depends on the sample and the species of phytoplankton. It is also necessary for the stabilization of both the pigment state and the thylakoid state in the dark. Another precaution that should be taken when using *in vivo* chlorophyll *a* fluorescence is excitation with moderate intensity light because at high irradiance levels fluorescence is quenched (Falkowski and Kiefer, 1985). Despite more than fifty years of research on chlorophyll fluorescence and the development of sound theoretical models describing variable fluorescence, there are many environmental factors that can affect fluorescence and of which we know little. Fluorescence is a powerful analytical technique since it can give information on photophysical processess and photosynthetic electron transfer in plants (Hipkins and Baker, 1986). Changes in these processess are caused by irradiance (Heany, 1978; Horton *et al.*, 1990), temperature, air pollution (Arndt, 1972), nutrients and chemicals that have invaded the environment such as pesticides and heavy metals in the water (Delcourt and Mestre, 1978; Benecke *et al.*, 1982, Bastian and Toetz, 1985; Zung *et al.*, 1990).

The major advantage of the bioassay technique described in this study are, however, the relative ease in which the measurements are made, the control over environmental conditions and background knowledge on the test organisms. Results could also be obtained within seconds as can be seen from the response of the algae to atrazine (Figure 4.6) and phenol (Figure 4.8). The bioassay technique is recommendable in spite of the lack of positive results as far as the reaction to heavy metals is concerned. This problem could possibly be solved through further studies.

CHAPTER 5

EVALUATION OF A SHORT-INCUBATION-TIME SMALL-VOLUME RADIOCARBON-UPTAKE ALGAL TOXICITY TEST

Introduction

Short term measurements of ^{14}C-assimilation by microalgae are seldom used as a method for toxicity testing in aquatic environments. The duration of the ^{14}C-assimilation test is normally a few hours (Nyholm and Damgaard, 1990) and used mostly with natural phytoplankton and natural water incubated at ambient temperatures and at high or saturating light intensities (Eloranta and Halttunen-Keyriläinen, 1984; Kuivasniemi *et al.*, 1985; Kusk and Nyholm, 1988, 1991). The use of cultured algae as test organisms in ^{14}C-uptake seems to be more rare (Kusk and Nyholm, 1988, 1991; Nyholm and Damgaard, 1990).

The technique used for the ^{14}C-assimilation test is similar to the one used for measuring phytoplankton primary production in laboratory incubators (Rai and Krambeck, 1992) by means of the ^{14}C-method (Steemann Nielsen, 1952). Lewis and Smith (1983) developed a small volume (1 ml), short-incubation-time (20 minutes) method for measuring algal photosynthetic rates as a function of incident irradiance. This method has proved to be useful both in the laboratory and in the field for the analysis of short term responses of algae to changes in available light. Any compound which might influence the ^{14}C-uptake rates of microalgae, at a particular irradiance, should give an indication of the potential toxicity of a particular substance. In this study the ^{14}C small-volume short-incubation time method was used to assess the potential toxicity of a few selected compounds.

Material and methods

Selenastrum capricornutum (CCAP 278/4) were used as the test organism and its culture conditions are described in Chapter 2.

Apparatus - A Photosynthetron (Figure 5.1), which was developed separately but which is similar to the "PHOTOTRON" as described by Rai and Krambeck (1992) was build by the Instrumentation Division of the University of the Orange Free State, Bloemfontein. The Photosynthetron consist of:

An incubation chamber with 16 metallic tubes made of stainless steel (No 6 in Figure 5.1). This chamber is connected to a constant temperature water bath (not shown in Figure 5.1) fitted with an immersion thermostat and automatic water circulating and temperature regulating systems (Grant Instruments, Cambridge, Ltd). Extractor fans (Torin TA450S), were mounted on one side of the lamp housing (made of aluminium) to serve as ventilators to remove heat, generated from the halogen lamps, between the incubation chamber and the light source. A glass cuvette, 13 mm in height made of 2 mm thick glass, was placed between the incubation chamber and light source and connected to the incubation chamber and water bath. Cooling water from the water bath was circulated through the incubation chamber and glass cuvette to maintain a constant temperature (23 ± 2 °C) in the sample cups. Standard glass scintillation vials were used to incubate the ^{14}C-enriched algal samples in. A perforated bakelite plate (2 mm thick) was placed between the light source and glass cuvette. The bakelite plate was perforated with 14 different mesh sizes to facilitate a series of irradiancies. Two of the tubes received no light and served as the controls. Illumination was supplied by 12 V, 50 W tungsten halogen lamps (Thorn, Model M 50 EXZ) mounted under each tube opening. The photosynthetron was placed on a shaking table to keep samples homogeneously mixed during the course of an experiment.

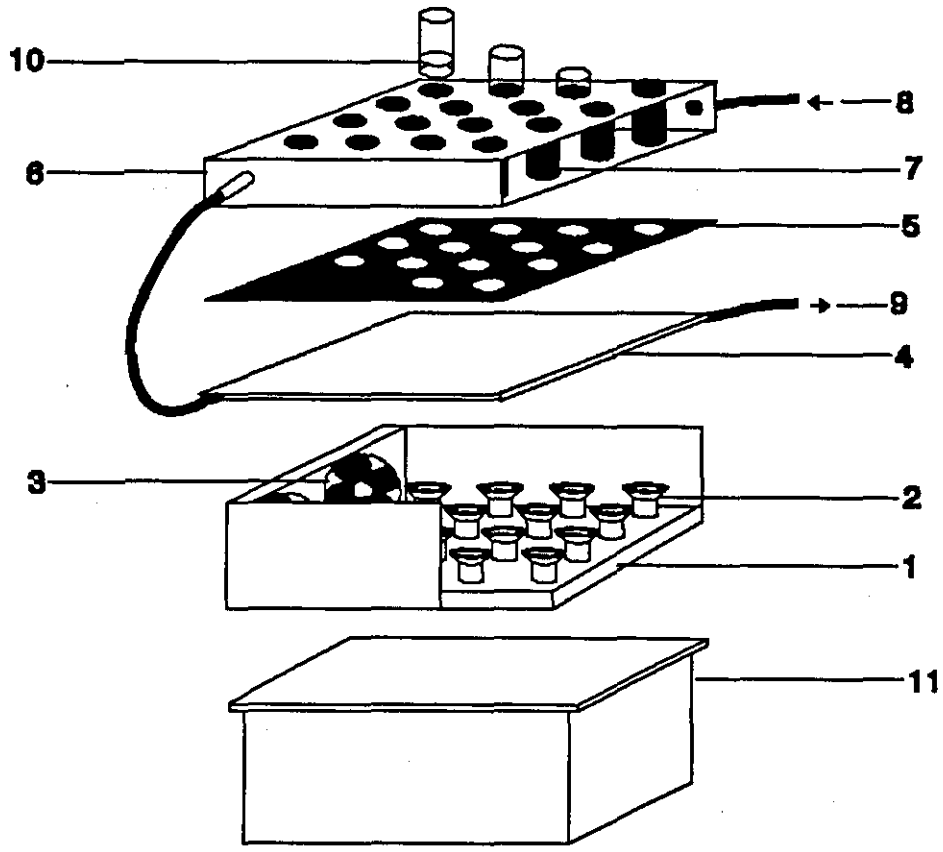


Fig. 5.1. Schematic diagram of the Photosynthetron with necessary instrumentation (not to scale). 1 - Light source , 2 - Tungsten halogen lamp, 3 - Extractor fans, 4 - Glass cuvette, 5 - Bakelite plate with perforations, 6 - Incubation chamber with metallic tubes, 7 - Metallic tube, 8 - Cooling water inlet, 9 - Cooling water outlet, 10 - Scintillation vial (sample cup), 11 - Shaking table.

Test procedure - The experimental procedure essentially followed the small volume short-incubation time method described by Lewis and Smith (1983). However, in this study 420 ml algal culture were divided into seven homogeneous 60 ml subsamples (control plus six different concentrations of substances) to which a toxin at different concentrations (Table 2.1, Chapter 2) and distilled H_2O as control, were added. These were then further divided into three samples of 20 ml each which were incubated for 30 min, 1 h and 4 h respectively. ^{14}C was added to one each of these subsamples to give a final specific activity of $\approx$ 0.05 µCi ml^{-1} in the cultures. After thoroughly stirring, by hand, 1 ml aliquots were dispensed by repetitive pipetting into scintillation vials and placed in the photosynthetron's incubation chamber.

Since I_k values are the transition between light dependent and light saturated photosynthesis, we reasoned that any factor affecting the algae would have a marked effect on the photosynthetic rates at the I_k irradiances. This also allowed us to measure many samples and treatments at any given time.

The I_k irradiance of the test algae was determined prior to the experiments from P/I curves using the perforated plate in the photosynthetron. Irradiance measurements were made with a Li-Cor Quantum Meter, Model LI-185B equipped with a LI-190SB quantum sensor placed inside the sample cups. In the construction of the P/I curves the photosynthetic rates (P) were taken as the DPM's at a particular irradiance (I). From the P/I curves, I_k was determined as the ratio of Pm/α, where Pm is the maximum ^{14}C-uptake rate taken as the constant DPM value in the light saturated part of the P/I curve. The slope of the linear part of the P/I curve, α, was determined by linear regression analysis using the DPM values in the irradiance range from 0 to 150 µmol quanta m^{-2} s^{-1}. For *Selenastrum capricornutum* I_k values of 250 to 320 µmol quanta m^{-2} s^{-1} were determined.

Two control vials with algae were placed in the dark (covered with aluminium foil) in the incubator to measure possible dark ^{14}C-assimilation. The incubation period lasted 30 min (which is also 30 min exposure to toxin). Secondly, all incubations were done at 250 to 320 mol quanta m^{-2} s^{-1}, which is the irradiance at the onset of light saturation (I_k). After incubation the samples were removed and placed on a shaker table in a fume cupboard and acidified with 0.5 ml 6N HCl. This was done to remove extra-cellular inorganic C and about 1 h was needed for complete C removal. The samples were then neutralized with 0.5 ml 6N NaOH and 10 ml Insta Gel scintillation liquid (Packard Instruments) was added directly into vials prior to counting on a LKB liquid scintillation counter (LKB Rackbeta, Model 1217, Liquid Scintillation Counter). The uptake rates as disintegrations per minute were corrected by subtracting the dark DPM's (DPM [light] - DPM [dark]). This value was used as the response parameter. Results were calculated as corrected DPM values (measured response) relative to the control DPM (control response) in distilled water. See Chapter 2, paragraph 2.2 for calculations and further treatments of data. After 1 h and 4 h of exposure to toxin the samples were again treated and incubated as described above. This procedure was repeated in duplicate for each toxic substance tested as well as a sample of treated sewage effluent (Chapter 2, paragraph 2.1).

Results

Figure 5.2 shows the influence of copper on ^{14}C-assimilation as measured after 30 min, 1 h and 4 h exposure. A progressive inhibition of photosynthesis can be seen with time. After 30 min exposure no inhibition was seen and the first inhibition was observed after 1 h exposure at concentrations > 1 mg l^{-1}. A 50 % inhibition (relative response of 0.5) was observed at a concentration of 2 mg l^{-1} copper. After 4 h exposure, ^{14}C-uptake was inhibited at concentrations of > 0.2 mg l^{-1} copper with a relative response of 0.9 (10 % inhibition). This inhibitory trend continued exponentially with increasing copper concentration to give 90 % inhibition when exposed to a concentration of 2 mg l^{-1}.

The dose-response curve of *Selenastrum* treated with cadmium (Figure 5.3) showed a high sensitivity of the test organism. The inhibition was almost linear with an increase in the concentration of cadmium with little difference between the three different incubation times. After 4 h of exposure the relative response was almost zero, which means 100 % inhibition.

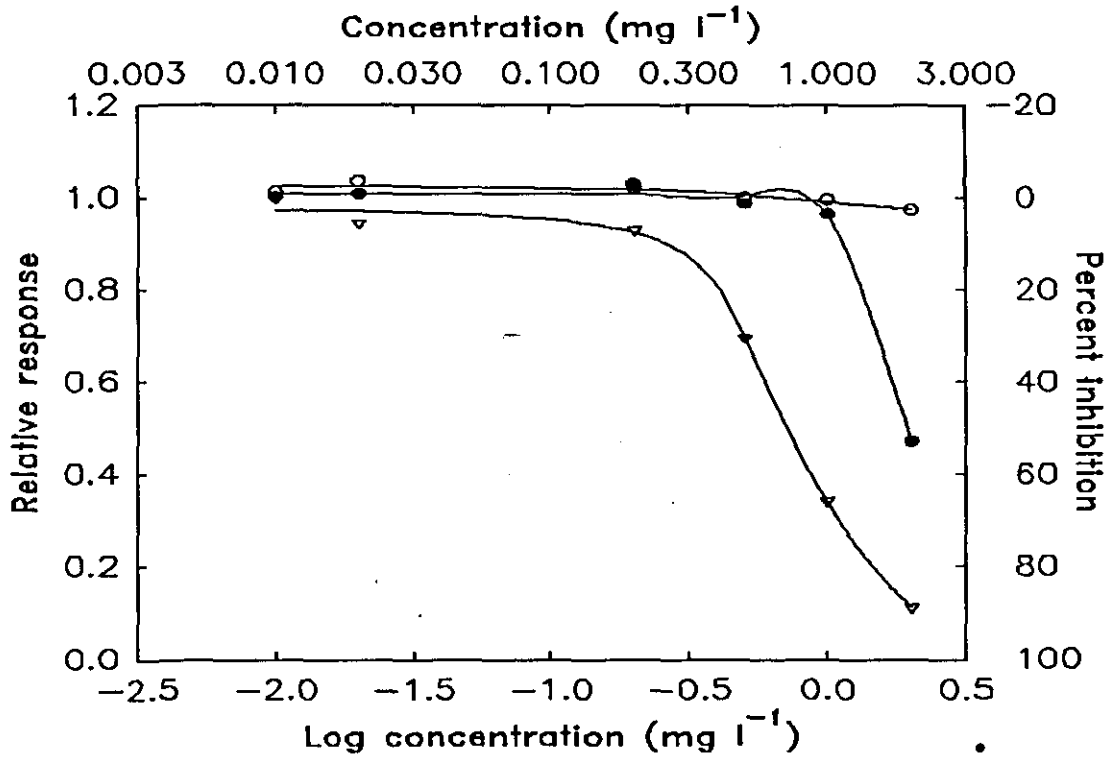


Fig 5.2. Dose-response curves for copper obtained with *Selenastrum capricornutum* in a short term ^{14}C-assimilation test. Response was measured after 30 min (○), 1 h (●) and 4 h (▽).

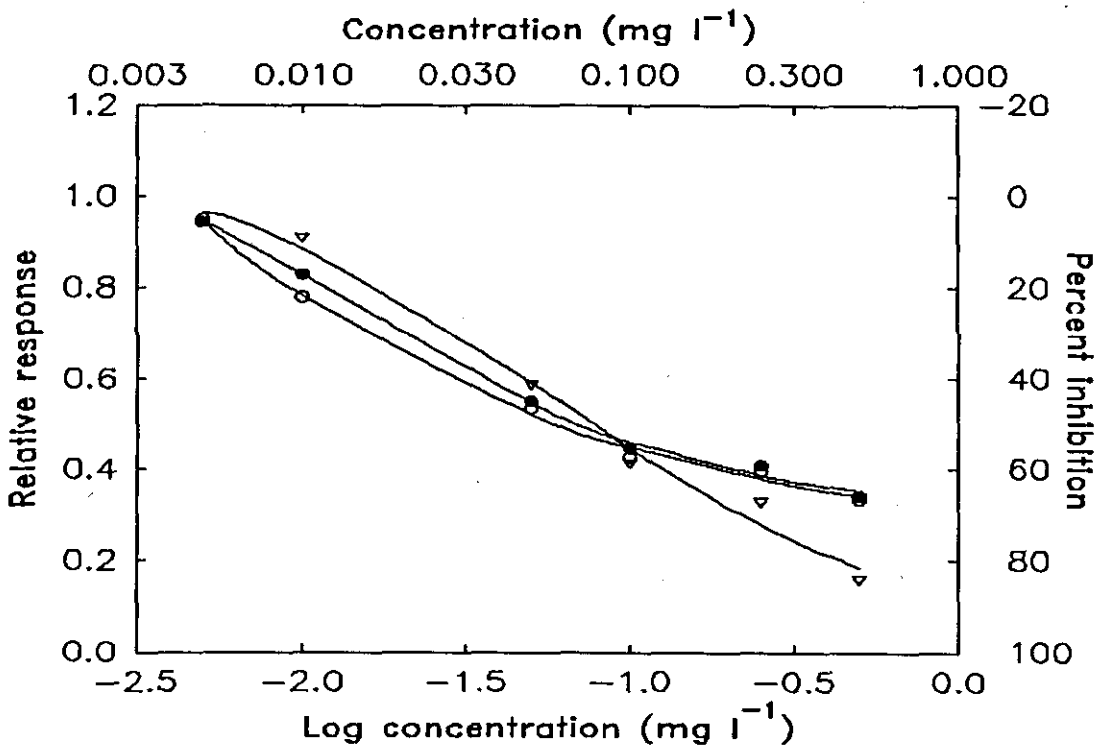


Fig 5.3. Dose-response curves for cadmium obtained with *Selenastrum capricornutum* in a short term ^{14}C-assimilation test. Response was measured after 30 min (○), 1 h (●) and 4 h (▽).

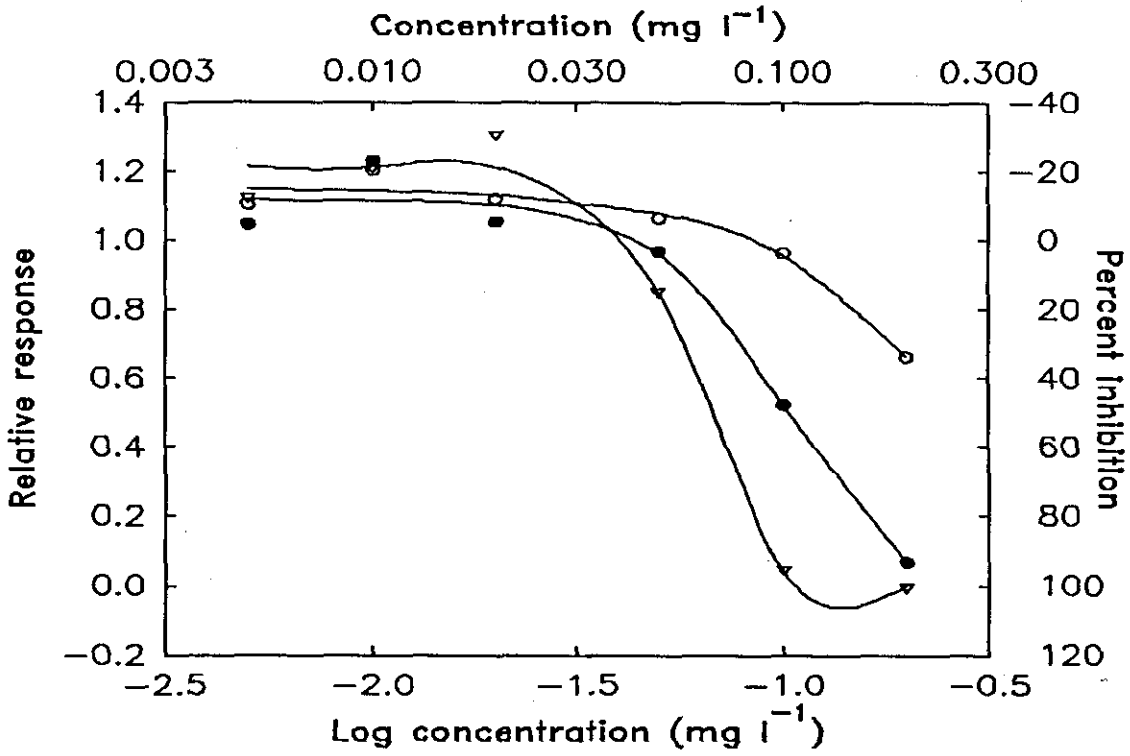


Fig 5.4. Dose-response curves for mercury obtained with *Selenastrum capricornutum* in a short term ^{14}C-assimilation test. Response was measured after 30 min (○), 1 h (●) and 4 h (▽).

Mercury toxicity increased with exposure time and concentration (Figure 5.4). There is, however, a stimulation in response of 20 % at low concentrations (0.005 to 0.02 mg l^{-1} mercury) after a 4 h exposure. The stimulation was somewhat lower (10 %) after 30 min and 1 h exposures. After this initial stimulation ^{14}C-uptake was inhibited almost 10 % when exposed to 0.05 mg l^{-1} mercury and almost 20 % at this same concentration after 1 h and 4 h respectively. At a concentration of 0.1 mg l^{-1} the inhibition was almost 10 % after

30 min, 50 % after 1 h and 100 % after 4 h. Inhibition increased to 30 % after 30 min, 90 % after 1 h and 100 % after 4 h exposure at a concentration of 0.5 mg l^{-1}.

Atrazine (Figure 5.5) showed a linear inhibition from 0.005 to 1 mg l^{-1} concentration where the inhibition was 100 %. ^{14}C-uptake was completely inhibited at atrazine concentrations > 1 mg l^{-1}. Little difference was seen between an exposure of 30 min, 1 h and 4 h.

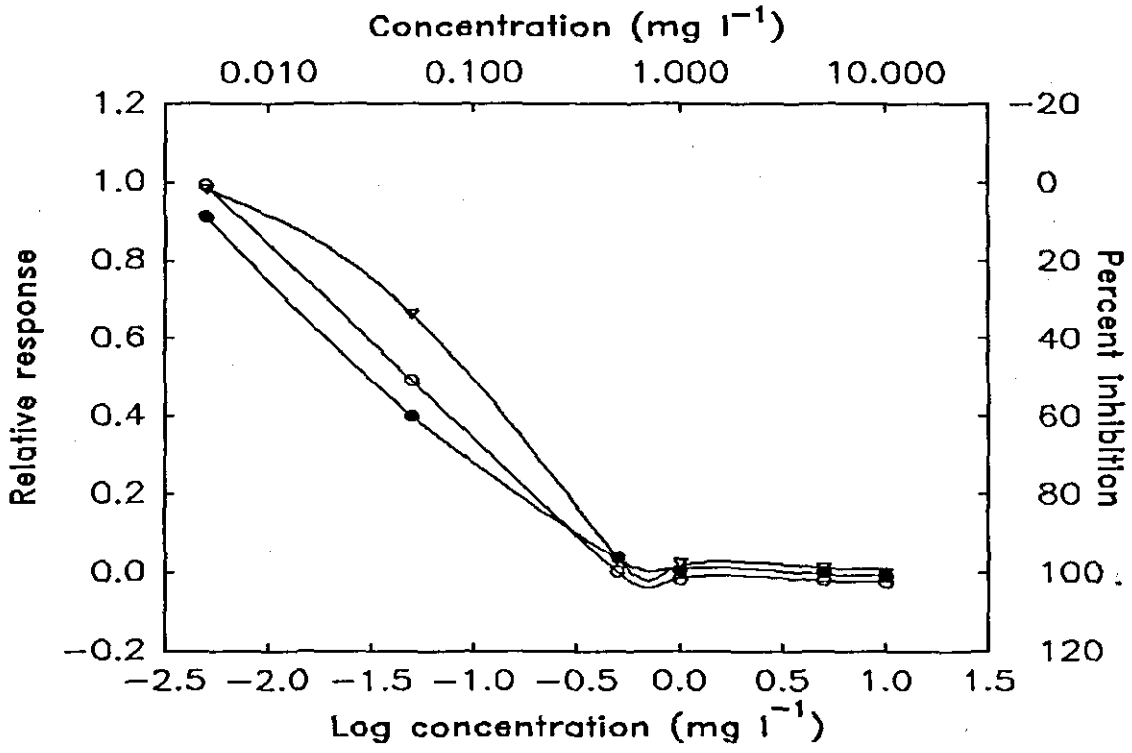


Fig 5.5. Dose-response curves for atrazine obtained with *Selenastrum capricornutum* in a short term ^{14}C-assimilation test. Response was measured after 30 min (○), 1 h (●) and 4 h (▽).

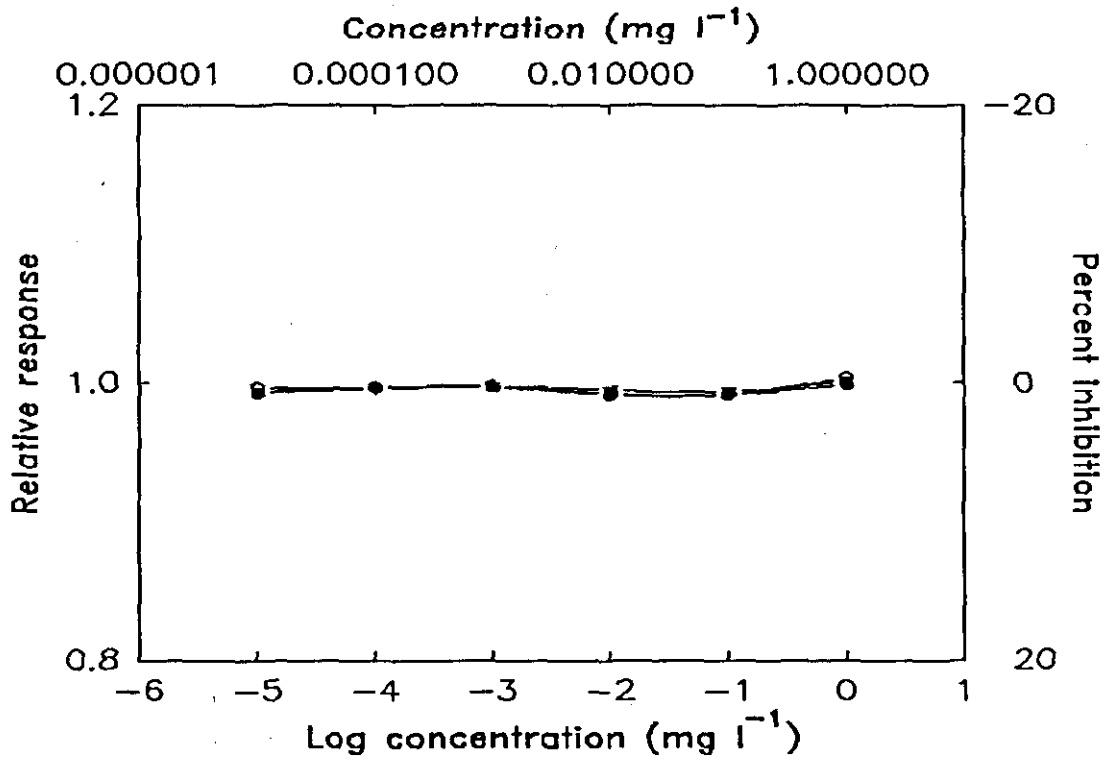


Fig 5.6. Dose-response curves for gusathion obtained with *Selenastrum capricornutum* in a short term ^{14}C-assimilation test. Response was measured after 30 min (○), 1 h (●) and 4 h (▽).

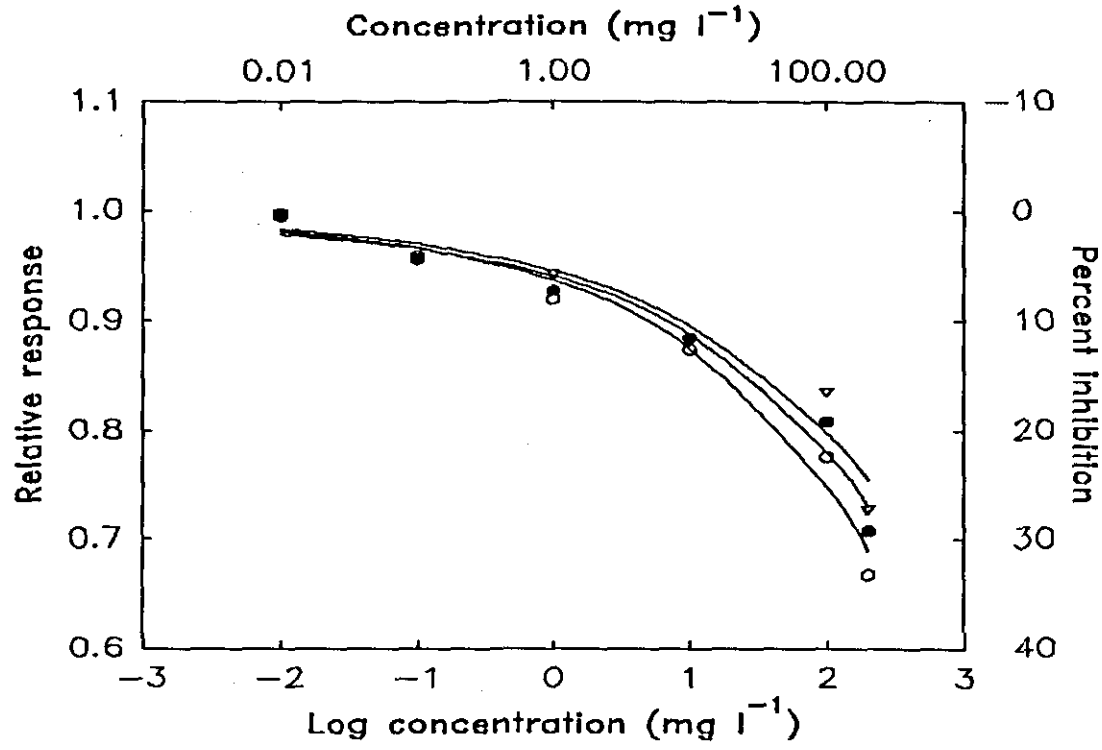


Fig 5.7. Dose-response curves for phenol obtained with *Selenastrum capricornutum* in a short term ^{14}C-assimilation test. Response was measured after 30 min (○), 1 h (●) and 4 h (▽).

The organophosphate gusathion had no effect on ^{14}C-uptake as can be seen from Figure 5.6. Phenol inhibited ^{14}C-uptake as can be seen in Figure 5.7. With an increase from 0.01 to 10 mg l^{-1}, ^{14}C-uptake was inhibited by about 12 % (relative response decreased from 1 to almost 0.875). A further increase in the concentration of phenol to 200 mg l^{-1} resulted in a relative response of almost 0.7 i.e an inhibition of about 30 %.

Table 5.1. Summary of the results from short-term ^{14}C-assimilation inhibition tests using *Selenastrum capricornutum* as test organism. The EC-values are in mg l^{-1}.

Compound	Time	EC10	EC50	EC90
Copper	30 min	*nr	nr	nr
	1h	1.264	1.968	2.890
	4h	0.246	0.736	2.134
Cadmium	30 min	0.006	0.060	nr
	1h	0.007	0.071	nr
	4h	0.009	0.077	1.371
Mercury	30 min	0.120	nr	nr
	1h	0.059	0.103	nr
	4h	0.049	0.061	0.085
Atrazine	30 min	0.013	0.049	0.176
	1h	0.008	0.036	0.218
	4h	0.021	0.073	0.262
Gusathion	30 min	nr	nr	nr
	1h	nr	nr	nr
	4h	nr	nr	nr
Phenol	30 min	4.716	nr	nr
	1h	6.657	nr	nr
	4h	9.541	nr	nr

*nr = no response

Tertiary treated municipal sewage effluent at concentrations of 25, 50, 75 and 100 % had no effect on ^{14}C-assimilation rates of *Selenastrum*.

Discussion

The results clearly show that short-term ^{14}C-uptake measurements can be used to determine the toxicity of certain compounds. Nyholm and Damgaard (1990) reported an EC10 value of 0.055, EC50 of 0.250 and EC90 of 2.0 mg l^{-1} for copper after 6 h ^{14}C-assimilation inhibition tests with *Selenastrum capricornutum*. Our EC10 and EC50 values of 0.246 and 0.736 mg l^{-1} were much higher than theirs but our EC90 values were similar after 4 h exposure (Table 5.1). The reason for their greater sensitivity can most probably be attributed to their longer exposure time, as our results clearly showed a progressive increase in inhibition with time (Figure 5.2). Furthermore, it is clear that 2 mg l^{-1} copper is lethal to algae irrespective of the exposure time.

The ^{14}C method of determining productivity assumes respiration in both light and dark bottles to be approximately equal. Reassimilation of intracellular CO_2 as a source of endogenous carbon during photosynthesis may also occur (Raven, 1972) and this process is affected by atrazine. According to Moreland (1980) atrazine affects the photosynthetic process in all algae in a similar way, that is by blockage of the electron transport in PSII through binding with its Q_B protein. This blockage in electron transfer results in the high inhibitory effect of atrazine on *Selenastrum* in ^{14}C-assimilation toxicity tests as shown in Figure 5.5.

The immediate ^{14}C-uptake response of the test organism to the atrazine concentrations tested is consistent with findings of researchers who exposed isolated algal species to atrazine in the laboratory. Larsen *et al.* (1986) reported an EC50 value of 42 µg l^{-1} for atrazine after exposing *Selenastrum* for 24 h to the toxicant. This value agrees with our EC50 value of 49 µg l^{-1} atrazine determined after 30 min exposure (Table 5.1). According to Eloranta and Halttunen-Keyriläinen (1985) the response of phytoplankton to shock-discharges should be studied only in short term (incubation time of 4 - 6 h) tests. The fact that our results were obtained after an incubation period of only 30 min, and the EC50 correlates well with 24 h incubation time of Larsen *et al*, (1986), implies a great saving in time, when the ^{14}C-uptake method is used, making it ideal in situations when a quick response is required.

Because the test duration using ^{14}C-uptake, is restricted to a few hours the test is very suitable for testing volatiles such as phenol (Nyholm and Damgaard, 1990). Although, only EC10 values were obtained in this study, indications from Figure 5.7 are that it would be possible to determine EC50 and EC90 values at higher concentrations. The EC10 value of 9.5 mg l^{-1} (Table 5.1) after 4 h exposure to phenol does, however, differ almost tenfold from the value of 110 mg phenol l^{-1} reported by Nyholm and Damgaard (1990) obtained by means of a 6 h ^{14}C-assimilation test. This means that our method is 10 times more sensitive in detecting phenol, which could be attributed to the shorter exposure time used.

Although it was possible to detect most of the compounds used within 30 min using ^{14}C-uptake, copper was only detected after 1 h exposure. The sensitivity of the short term ^{14}C-uptake toxicity test varied with time and also between substances. The heavy metals copper and mercury were most sensitive after a 4 h exposure, cadmium and phenol after 30 min and atrazine after 1 h. Volatile compounds such as phenol require short term exposure tests.

Potential hazardous chemicals could be detected within 30 min using the ^{14}C-assimilation algal toxicity test, which makes it well suited as a rapid screening bioassay technique. The short term ^{14}C-assimilation test for toxicants, complies with most prerequisites required for an acute test method i.e. it is rapid and easy to perform and is reproducible. This method could also be used in most laboratories equipped to do phytoplankton primary production measurements. However, cognizance should be taken of the fact that different compounds react differently in terms of their toxicity. Therefore, the advantages and limitations of each method should be considered when interpreting results. For example, a longer

incubation time is required to detect copper toxicity at low concentrations, whilst for compounds such as phenol, the incubation time should be as short as possible. A further complication is that certain compounds could initially stimulate algal growth at low concentrations. This is clearly shown by the results for mercury (Figure 5.4) and can be attributed to a transient physiological overcompensation in response to chemical stresses within the test species acclimation range. This response, known as hormesis, can be considered as being an integral part of stress response not only to toxicants but also to environmental pressures according to Laughlin *et al.* (1981).

CHAPTER 6

THE USE OF OXYGEN EVOLUTION TO ASSESS THE SHORT TERM EFFECTS OF TOXICANTS ON ALGAL PHOTOSYNTHETIC RATES

Introduction

Concern over environmental perturbations has resulted in the search for methods of evaluating the effects of introducing substances to aquatic systems that may disrupt the equilibrium of those systems. The response of organisms to pollutants (excessive nutrients or toxic substances) is central to the disruption of equilibria and it is because of this that bioassays are mostly used to evaluate the effects of pollutants.

In algal suspensions, changes in the dissolved oxygen content could be brought about either by photosynthetic liberation or respiratory depletion. From these changes two completely different metabolic pathways could be used in toxicity studies, i.e. either photosynthesis or respiration. Hostetter (1976) developed a rapid bioassay sensitive to nutrients and the presence of toxic substances using a Clark type oxygen electrode and a reaction chamber containing the water to be tested and the test organism. The rate of net photosynthesis as oxygen production was determined in this method. A prerequisite of such a method is that it is essential that the experimental conditions are reproducible. This implies precise control of environmental conditions such as temperature and the light field, together with a rapid reproducible assessment of O_2-evolution. Clark-type O_2-electrodes offer advantages over other methods in that they allow for continuous measurements of oxygen production or consumption (Dubinsky *et al.*, 1987).

Net photosynthesis can be inhibited by toxic concentrations of substances. In this study, results are presented where the oxygen chamber, as described by Dubinsky *et al.* (1987), was used to measure the possible effects of selected toxic substances (copper, cadmium, mercury, atrazine, gusathion and phenol; See Table 2.2, Chapter 2) on net photosynthetic rates as determined from oxygen liberation.

Material and methods

The test organisms and their culture conditions are described in Chapter 2.

Experimental procedure - The experimental setup was the same as described by Dubinsky *et al.*(1987). The O_2-chamber was irradiated from the front using an ordinary 35 mm slide projector. The measurements were all done at an irradiance equal to I_k, which is the irradiance at the onset of light saturation during photosynthesis.

I_k was from P/I curves measured prior to the toxicity tests and varied between 250 to 320 µmol quanta m^{-2} s^{-1}. P/I curves were determined at different irradiancies using neutral density filters placed between the projector and the chamber. Irradiance measurements were taken with a Li-Cor Quantum Meter, Model LI-185B equipped with a LI-190SB quantum sensor placed at the rear of the chamber. The oxygen rates (as µg O_2 l^{-1} min^{-1}) that were calculated from recorder traces were plotted against the irradiancies and from these plots I_k was determined (see Chapter 5 for determination of I_k).

A single measurement at a specific irradiance is desirable for the purpose of toxicity tests because of the time constraints of most other tests. Following this approach a sample could be tested in 30 minutes. I_k was chosen because this is the irradiance where a transition takes place from light limited to light saturated growth. We reasoned that any factor which could affect photosynthesis would be clearly seen at this irradiance (See also Chapter 5).

Algal samples were injected into the chamber through a hole at the top, that was closed with a pierced PVC screw cap during measurements. Toxic substances (See Table 2.2,

Chapter 2) were injected, during incubation, through the 1 mm diameter capillary in the screw cap. The samples were mixed by means of a glass covered magnetic spinbar driven by a magnetic stirrer. Oxygen was measured with a Clark type electrode mounted in the side of the cuvet so that the membrane covered end just extended into the chamber. This system was calibrated once a day against distilled water air equilibrated at the experimental temperature and against a zero oxygen solution (saturated Na_2SO_3 solution). The span in mV between zero oxygen (0 % O_2) and air saturated water (100 % O_2) was measured at the experimental temperature and used in the calculations. These changes in O_2 concentration were recorded on a Graphic 1002 Y/t chart recorder (Lloyd Instruments, Southampton) and expressed as mV min^{-1}. These traces were converted into µmol O_2 l^{-1} min^{-1} by means of calibration factors.

Calculations - At a temperature of 25 °C (which was the temperature in the chamber) and an altitude of 1244 m (Bloemfontein) the dissolved oxygen is 7224 µg O_2 l^{-1} (i.e. 8.4 mg O_2 l^{-1} at 25 °C multiplied by a correction factor of 0.86 for the altitude).

The mV span was converted into µg O_2 l^{-1}. For example: 100 % O_2 = 389 mV and 0 % O_2 = 4.2 mV

mV span = 384,8 mV = 7224 µg O_2 l^{-1}
1 mV = 18.773 µg O_2 l^{-1}

These factors were used to convert recorded traces of changes in O_2 concentration from mV min^{-1} into µg O_2 l^{-1} min^{-1} which were taken as the measured response. The results were expressed as a response relative to the control response, after calculation of dilution factors brought about by the addition of toxins directly to the sample. EC-values were calculated as described in Chapter 2.

Results

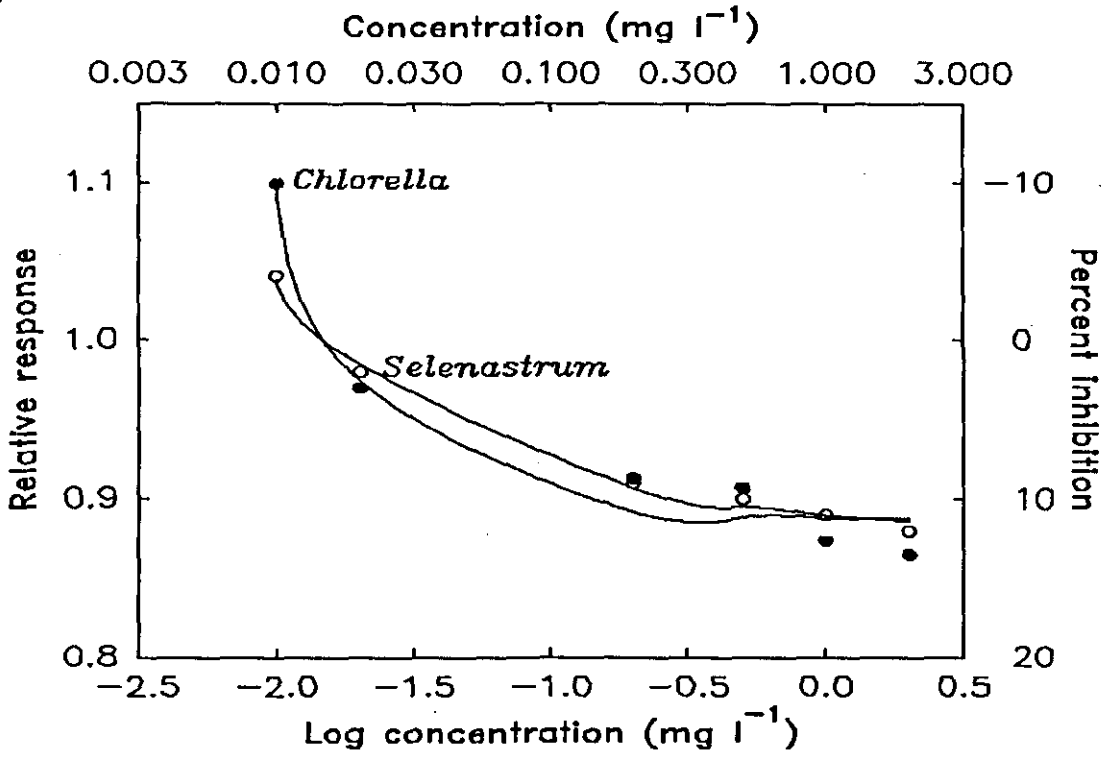


Fig 6.1. Dose-response curves of algae exposed to different concentrations of copper. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

From Figure 6.1 it can be seen that there was an initial stimulative response of 10 % and 5 % (relative response of 1.1 and 1.05 respectively) for *Chlorella* and for *Selenastrum* which were respectively measured at low concentrations (0.01 mg l^{-1}). After the initial stimulatory response there was a decline to 0.97 with a doubling of copper concentration from 0.01 to 0.02 mg l^{-1}. A further tenfold increase in the copper concentration resulted

in a relative response value of 0.92 (i.e. a further 5 % inhibition). At higher concentrations of 0.2 to 2 mg l^{-1} copper there was only a 3 % increase in response (0.90 to 0.87). The response of both species to copper was almost identical in the concentration range tested.

The effect of different concentrations of cadmium on oxygen evolution over a short exposure period to the heavy metal are shown in Figure 6.2. No initial stimulation was seen at the low concentrations as was the case with copper dosages (Figure 6.1). From an initial zero inhibition (1.0 relative response) at low concentrations ranging from 0.005 to 0.05 mg l^{-1} *Chlorella* exhibited a sharp inhibition of almost 25 % (relative response change of 0.25 from 1.0 to almost 0.75) when the concentration of cadmium was doubled to 0.1 mg l^{-1}. *Selenastrum*, at this same concentration, only experienced a 10 % inhibition. With a two and a half fold increase in the cadmium concentration to 0.25 mg l^{-1} the inhibition increased with a further 10 % and 5 % for *Chlorella* and for *Selenastrum* respectively, after which the inhibition remained constant at a relative response of 0.65 (35 % inhibition) and 0.85 (15 % inhibition) respectively for *Chlorella* and for *Selenastrum* at higher concentrations (0.25 and 0.5 mg l^{-1} cadmium).

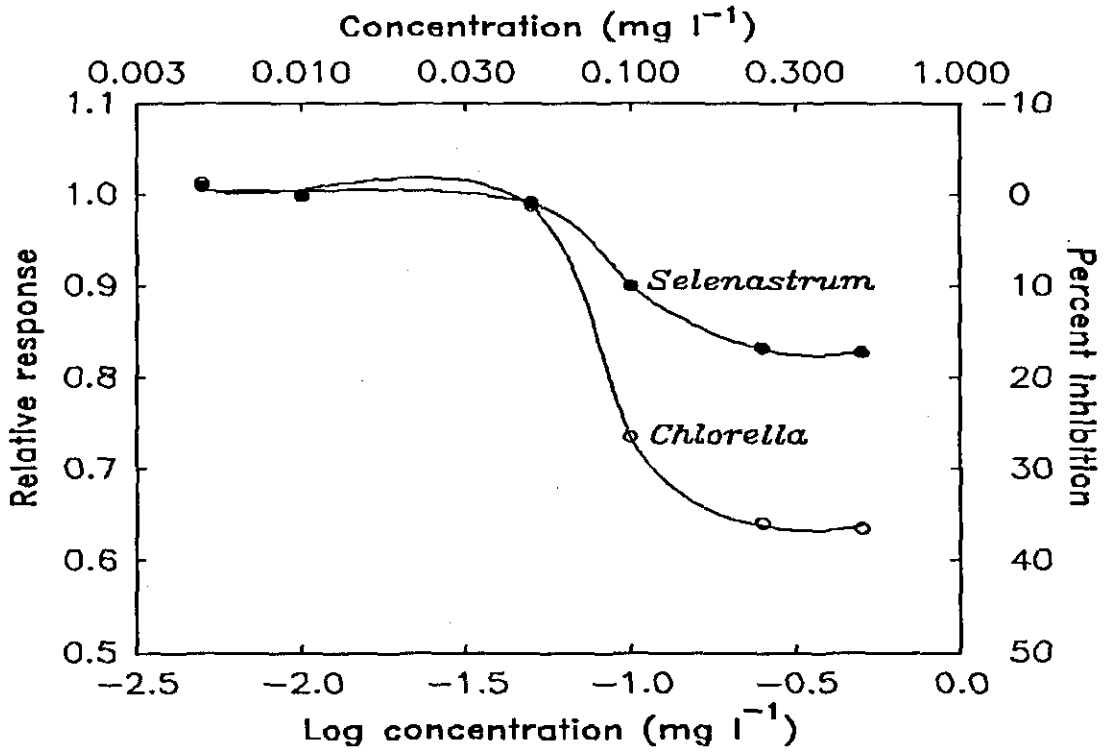


Fig 6.2. Dose-response curves of algae exposed to different concentrations of cadmium. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

Shown in Figure 6.3 is the effect of mercury on oxygen evolution. From an initial stimulation of 18 % at low concentrations of 0.005 mg l^{-1} mercury for *Selenastrum*, a linear relationship (r^2 = 0.9895) between log concentration and relative response was observed. An increase from 0.005 to 0.5 mg l^{-1} mercury resulted in a decrease in the relative response range of 1.2 to 0.5 (20 % stimulation to 50 % inhibition). *Chlorella* responded in a similar fashion excepting for stimulations at low concentrations (0.005 to 0.2 mg l^{-1} mercury).

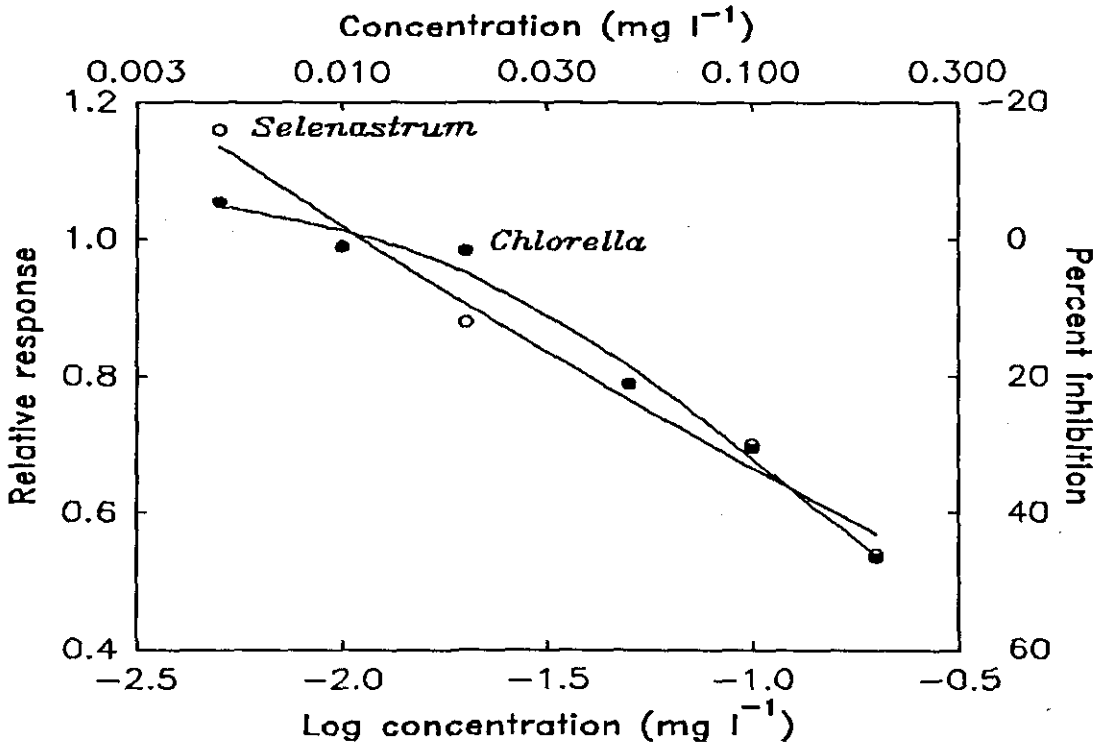


Fig 6.3. Dose-response curves of algae exposed to different concentrations of mercury. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

Figure 6.4 shows an inverse sigmoidal dose-response curve for atrazine. This means that at low concentrations of 0.005 and 0.05 mg l^{-1} the algae were unaffected. After this initial "no effects" phase there is an exponential inhibitory response which eventually ends in a constant phase at high concentrations (5 and 10 mg l^{-1}) where no further inhibition was seen. The relative response of *Selenastrum* ranged from 1 to 0 (i.e. 0 % inhibition to 100 % inhibition) whereas for *Chlorella* it ranged from 1 to almost -1 (i.e. 0 to 200 % inhibition).

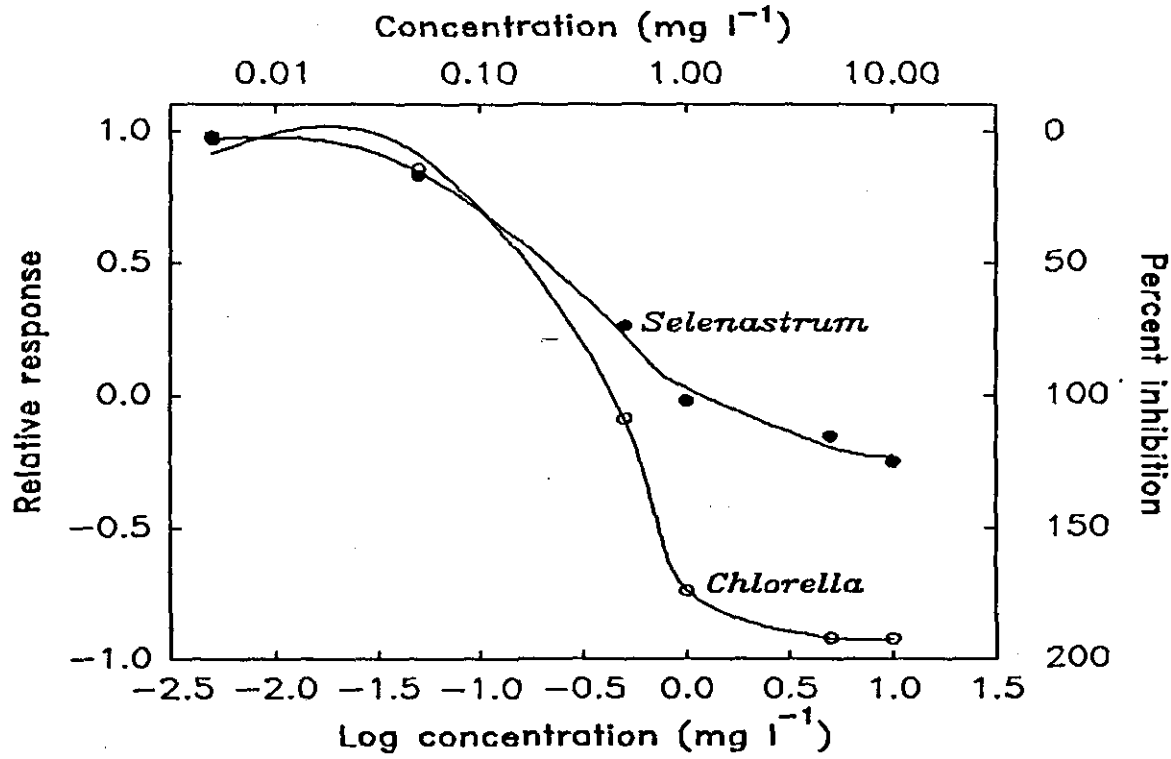


Fig 6.4. Dose-response curves of algae exposed to different concentrations of atrazine. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

The organophosphate gusathion had no effect on the oxygen evolution as can be seen from Figure 6.5. There is also no difference in the response of the two algae.

Shown in Figure 6.6 is the effect which phenol had on the oxygen evolution of *Selenastrum* and *Chlorella*. No inhibition was seen for *Selenastrum* at a low concentration of 0.01 mg l^{-1} whereafter it increased to a concentration of 1 mg l^{-1}. The inhibition remained constant at a relative response of 0.7 for concentrations of 1 to 200 mg l^{-1} phenol. *Chlorella* also exhibit an exponential decline (increase in inhibition) at low concentrations dosages, but the only difference is that this stabilized at 1 mg l^{-1} and higher. The maximum inhibition of 30 % was the same for both algae.

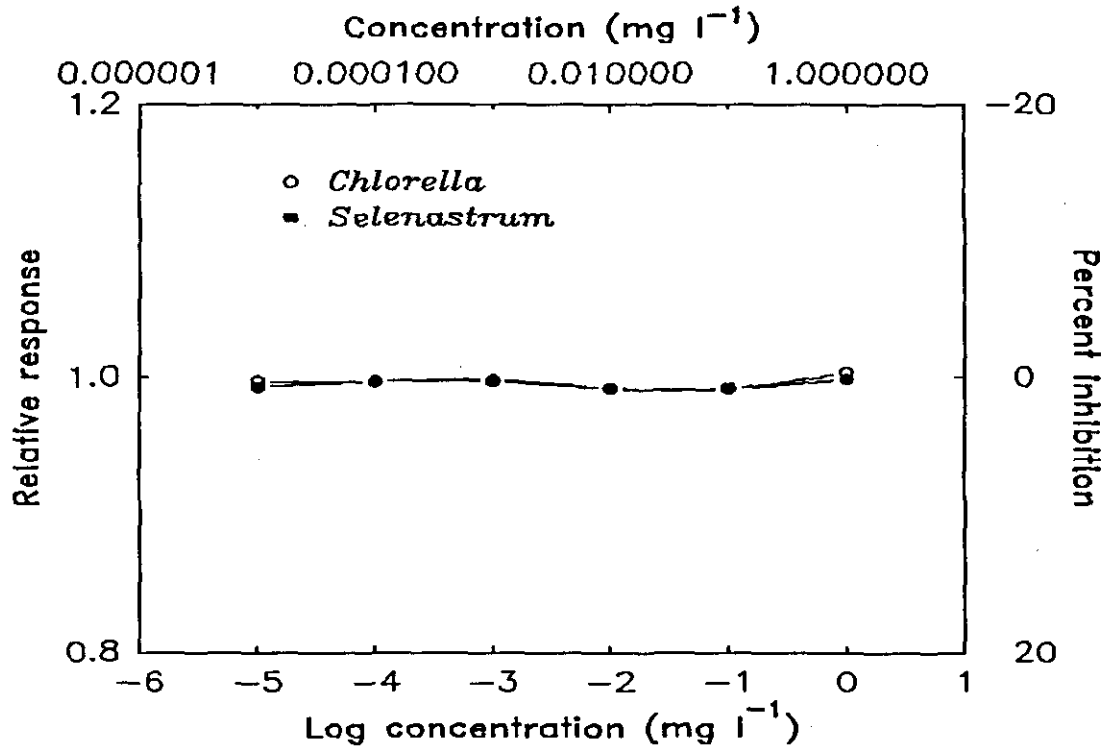


Fig 6.5. Dose-response curves of algae exposed to different concentrations of gusathion. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

Table 6.1. Summary of the results from O_2-evolution toxicity experiments expressed as EC values in mg l^{-1}.

Compound	*Selenastrum*			*Chlorella*		
	EC10	EC50	EC90	EC10	EC50	EC90
Copper	0.280	*nr	nr	0.147	nr	nr
Cadmium	0.100	nr	nr	0.072	nr	nr
Mercury	0.021	nr	nr	0.030	nr	nr
Atrazine	0.031	0.222	0.751	0.086	0.305	0.433
Gusathion	nr	nr	nr	nr	nr	nr
Phenol	0.076	nr	nr	0.143	nr	nr

*nr = no response

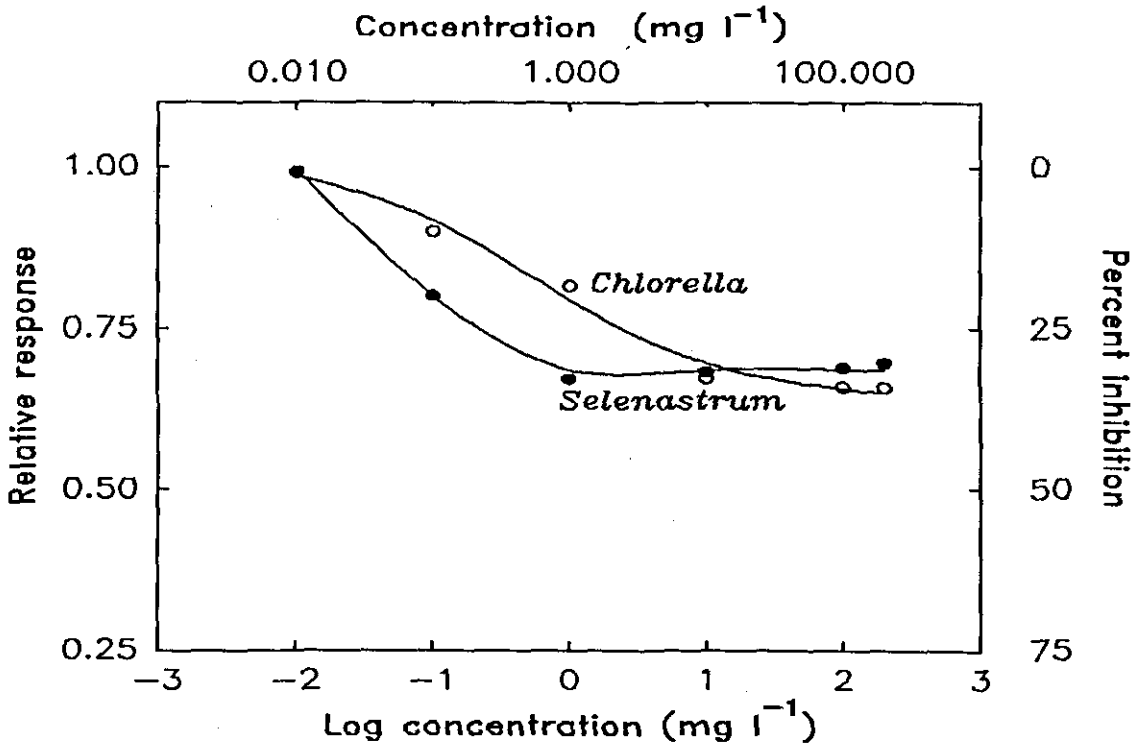


Fig 6.6. Dose-response curves of algae exposed to different concentrations of phenol. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

Tertiary treated municipal sewage effluent at 25, 50, 75 and 100 % concentrations did not inhibit algal oxygen evolution.

Discussion

The results show clearly that oxygen production, as measured in the oxygen chamber (Dubinsky *et al.*, 1987), was affected by some of the compounds tested for their potential toxicity. From the various EC-values as shown in Table 6.1 it can be seen that EC50 values were found only for atrazine and mercury over the concentration ranges tested. The same is seen for the EC90 values where *Selenastrum* appears to be slightly more sensitive to mercury than *Chlorella* because no EC90 value could be calculated for the latter at the tested concentrations. From the EC10 values it can be seen that *Selenastrum* seems to be more sensitive to atrazine and phenol than *Chlorella* (Figures 6.4 and 6.6; Table 6.1). *Chlorella* on the other hand seems to be more sensitive than *Selenastrum* to copper and cadmium (Figures 6.1 and 6.2; Table 6.1) in terms of the EC10 values.

The greater inhibition, as caused by Hg compared to Cu and Cd, can be ascribed to the interruption of electron flow by Hg at multiple sites such as plastocyanin (Katoh and Takamiya, 1964), the reaction centre of photosystem I (Kojima *et al.*, 1987) and iron sulphur centres (Gelbeck *et al.*, 1977). It is also known that the multiple effects of mercury arise from its interaction with the sulphydryl groups of protein (Passow *et al.*, 1961). According to Shioi *et al.* (1978) copper directly inactivated the ferrodoxin and reduced the $NADP^+$ photoreduction in the electron transport of photosynthesis. A secondary and less severe inhibitory effect of copper is in photosystem II (PSII) between the oxidizing side of the reaction centre of PSII and the electron donating site of DPC.

Rai *et al.*, (1991) suggested, in a study on the toxicity of Hg and Zn to *Chlorella*, that Hg has a greater toxic potential than Zn. Its great penetration capacity into the biological membrane, and multiple binding with the electron transport system coupled with ATP generation seems to be the major cause for the inhibition of all biological activities. They also pointed out that PSII was the primary site of action of Hg in *Chlorella vulgaris*. Hg, Cu and Cd impair the electron transport system and because of this ATP production, which in turn is needed in the Calvin cycle, is reduced. Thus overall cell

metabolism is influenced and, therefore, O_2-production is affected. This chain of events would possibly apply to all eucaryotic algae.

At EC50 and EC90 levels the response of *Selenastrum* and *Chlorella* to atrazine toxicity is opposite to the response as determined at the EC10 level. *Chlorella* is more sensitive than *Selenastrum* to high atrazine concentrations, but *Selenastrum* is more sensitive than *Chlorella* at the EC10 level. The drastic inhibition of photosynthesis as caused by atrazine gives direct evidence for the inhibition of the Hill reaction (PSII) by this herbicide. The inhibition by atrazine is similar to that by DCMU according to Singh *et al.* (1983).

The chemical characteristics of the algal growth medium might be an important determining factor of toxicity thresholds. Turbak *et al.*, (1986) reported an EC50 value from O_2-evolution assays of 69.7 $\mu g\ l^{-1}$ and when using natural water samples as growth medium an EC50 of 854 $\mu g\ l^{-1}$ was obtained for atrazine with *Selenastrum* as test organism. Our results gave an EC50 of 222 $\mu g\ l^{-1}$ for *Selenastrum* and 305 $\mu g\ l^{-1}$ for *Chlorella.* These results support observed variances obtained by different labs with identical test organisms and different methods (Nyholm and Källqvist, 1989).

Gusathion had no effect on the photosynthetic rates of either test organisms. The reason for this is that gusathion is an organophosphate and it is known that organophosphates could be utilized as phosphate source by algae. The toxicity of phenols occur by virtue of their oxidation to the corresponding quinones, which can readily oxidize sulphydryl groups on enzymes, thereby disrupting the normal oxidation-reduction balance in algae (Hanchey-Bauer, 1978). *Selenastrum* was almost twice as sensitive towards phenol than was *Chlorella* as can be seen from Table 6.1. No related data could be found to compare our data with in order to measure our method against what has already been done.

Treated sewage had no influence on algal O_2-production which indicates that no substance that could be harmful to O_2-evolution was present in the effluent.

The major disadvantage of the oxygen-evolution method, as it was applied in our tests, is that it measures only one part (photosynthesis) of one metabolic process, net photosynthesis, rather than growth which can be considered the result of numerous metabolic processes. The primary advantage of the rapid O_2-measurements, however, is that it allows for the determination of photosynthetic rates using small samples (15 ml) and results could be obtained within 5 minutes, after the chamber and sample had been stabilized.

CHAPTER 7

EVALUATION OF *DAPHNIA PULEX* AS TEST ORGANISM IN TOXICITY ASSESSMENT OF NATURAL WATER

Introduction

When selecting a bioassay procedure/method to evaluate water quality toxicity the choice of test organisms must be highly selective. According to Kenaga and Moolenaar (1979) water quality limitations, based on toxicity data for fish and daphnids, should be sufficiently restrictive to protect algae and macrophytes. The freshwater cladoceran, *Daphnia*, commonly known as the water flea has been used in freshwater toxicity studies for over a century (APHA, 1989) world wide and they are often used as standard toxicity tests. Toxicity tests using *Daphnia magna* became enshrined as the British standard (British Standard, 1983) when it was adopted by the Environment and Pollution Committee. Toxicity tests using animals are carried out with a limited number of test subjects each with an assumed individual tolerance toward the particular test material. The response recorded in animal tests is often quantitative or categorical (e.g. death) (Nyholm *et al.*, 1992).

Daphnia pulex was used as test organism to evaluate acute toxicity (48h) of the following substances; copper, cadmium, mercury, atrazine, gusathion and phenol (Table 2.1, Chapter 2). Samples with unknown toxicity were collected from tertiary treated municipal sewage effluent and were evaluated along with the other substances. The results obtained served as a reference base for comparisons with the algal bioassays (Chapters 3, 4, 5 and 6).

Material and methods

Test organisms and culture conditions - *Daphnia pulex* were obtained from the Hydrological Research Institute, Department of Water Affairs, Pretoria. The *Daphnia* were cultivated in moderately hard reconstituted water (Table 7.1) in 2 l glass beakers. The animals received three feedings weekly of a food suspension consisting of fish pellets (trout chow), alfalfa and yeast. Stock cultures were maintained under controlled conditions at a temperature of 20 ± 2 °C and a photoperiod of 16 h light and 8 h dark.

Table 7.1. Moderately-hard reconstituted water.

Reagent	Concentration (mg l^{-1} water)
$NaHCO_3$	96.0
$CaSO_4.2H_2O$	60.0
$MgSO_4.7H_2O$	123.3
KCl	4.0

Healthy individuals should be used in the toxicity tests and, therefore, the *Daphnia* were not taken from cultures that were producing ephippia (Truter, 1990). Adult females bearing embryos were removed from the stock cultures 24 h preceding the initiation of the tests and placed in 400 ml beakers with 300 ml medium under the same controlled conditions (light, temperature and feeding) as the stock cultures. The young that were found in the beaker the following day were used in the tests.

Test procedure - Five organisms (less than 24 h old) were placed in a 50 ml glass beaker containing 25 ml test solution. Each beaker were replicated four times. Moderately-hard water (Table 7.1) was the control solution. Test solutions were also diluted with moderately-hard water. The test conditions were the same as the culture conditions i.e.

20 ± 2 °C with 16h light and 8h dark, except that no feedings were given during test period. Mortality, that is, no movement of body or appendages on gentle prodding was recorded in each test vessel after 24 h and 48 h. During each observation dead organisms were removed.

Estimating LC values - The lethal concentration (LC) that causes 50 % mortality in test vessels (LC50) were estimated graphically (Figures 7.1 - 7.6). The toxin concentrations and the corresponding mortality percentage were plotted on semi-logarithmic graph paper. The two points separated by the 50 % mortality line was connected with a straight line. The value of the point where this line and the 50 % mortality line intersect was read from the graph and this is then the LC50 value for the test. LC10, that is the first observable effect, and LC90 was estimated in the same way (Truter, 1990). The results were used as a reference base to compare with algal assays, and the method was considered to be a standard assay method. The calculations were therefore made in accordance with the prescribed methodology (Truter, 1990).

Results

The response of *Daphnia* to copper toxicity are shown in Figure 7.1. Copper had a severe influence on the mortality of *Daphnia* where 80 % and more of all test organisms were dead after 24 h at Cu concentrations of 0.02 mg l^{-1}. No Cu toxicity was found at a concentration of 0.01 mg l^{-1} copper. Very little difference was found between an exposure of either 24 or of 48 h.

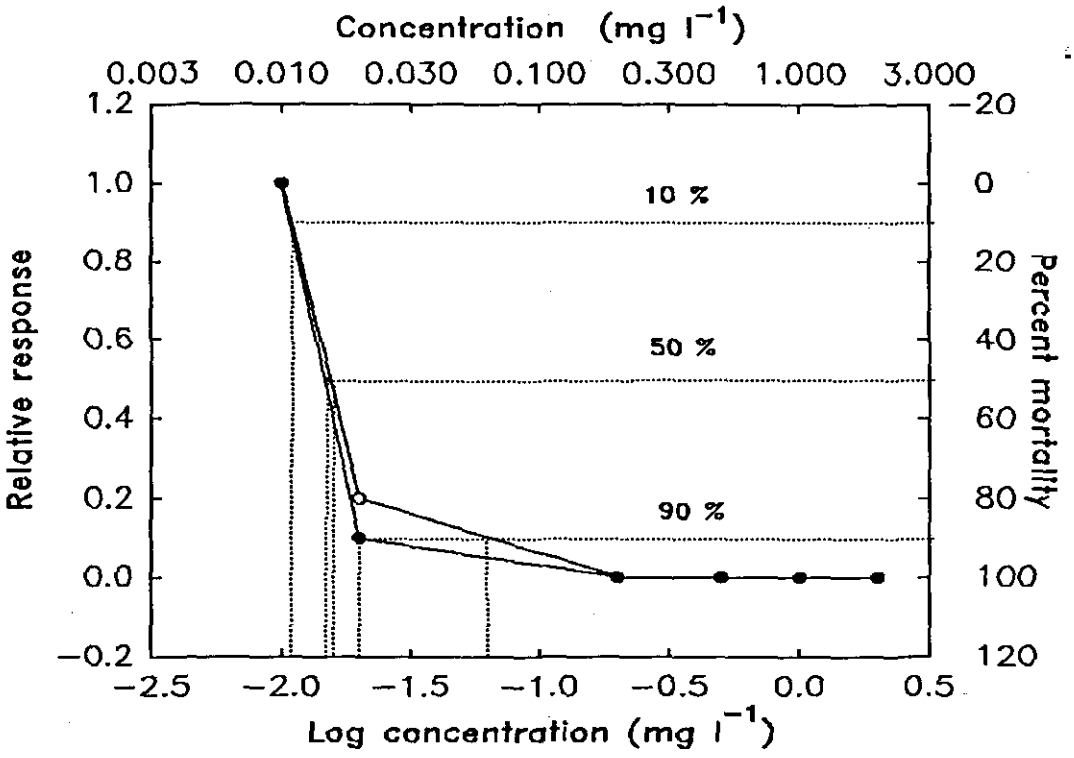


Fig 7.1. Percent mortality against different copper concentrations using *Daphnia pulex* as test organism; where (○) = 24 h and (●) = 48 h.

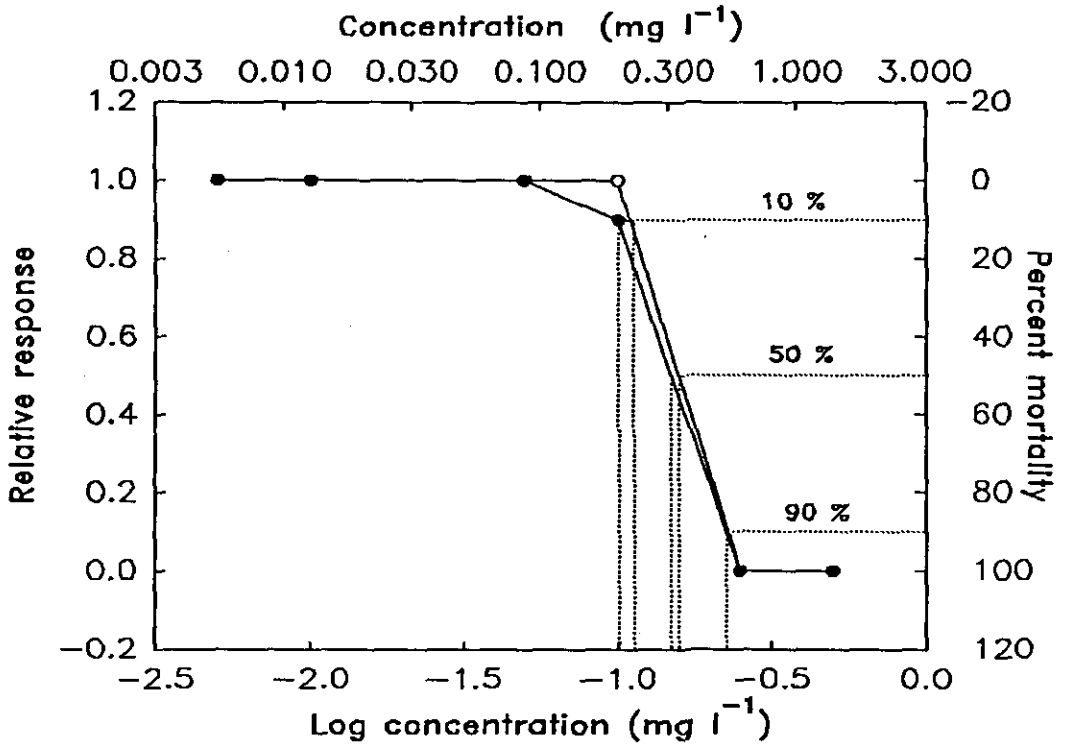


Fig 7.2. Percent mortality against different cadmium concentrations using *Daphnia pulex* as test organism; where (○) = 24 h and (●) = 48 h.

Cadmium at equivalent concentrations appeared to be less toxic than copper, as shown in Figure 7.2. The first indication of cadmium toxicity was observed at a concentration of 0.1 mg l^{-1} after 48 h exposure where 10 % mortality occurred. A fivefold increase in cadmium concentration to 0.5 mg l^{-1} resulted in a 100 % mortality rate after 24 h and 48 h of exposure.

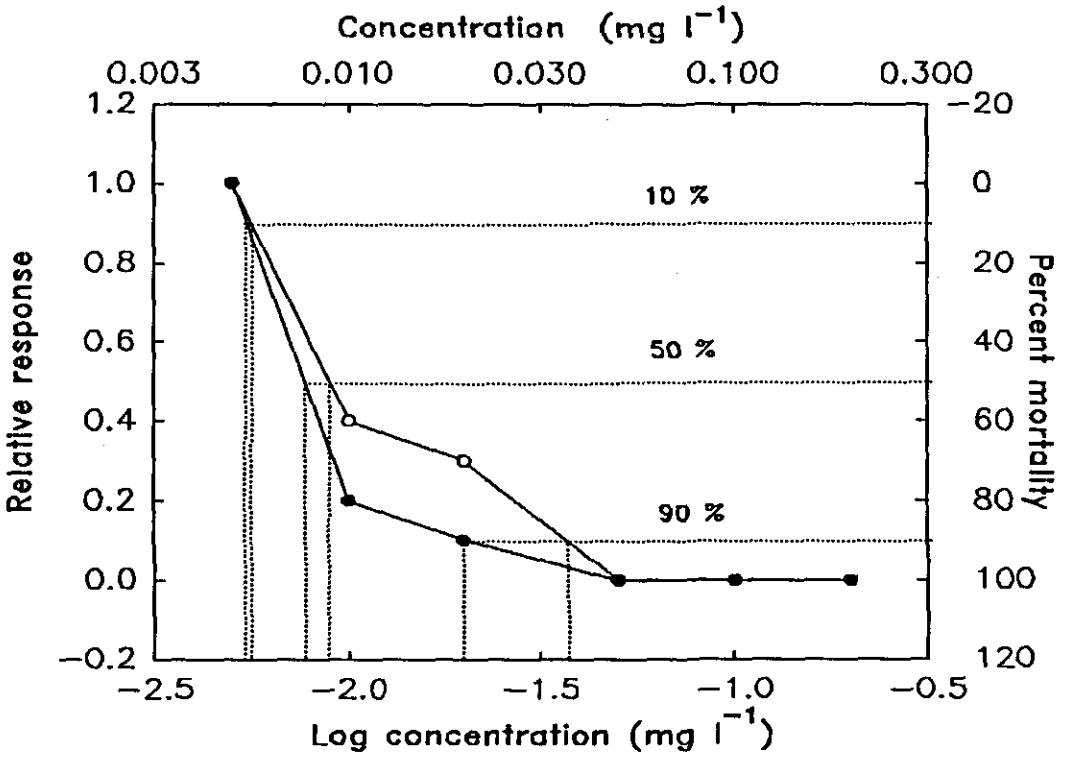


Fig 7.3. Percent mortality against different mercury concentrations using *Daphnia pulex* as test organism; where (○) = 24 h and (●) = 48 h.

Mercury has a high toxicity as can be seen in Figure 7.3. At a mercury concentration of 0.01 mg l^{-1} 60 % of the organisms were killed after 24 h of exposure and 80 % after 48 h. At a concentration of 0.02 mg l^{-1} the mortality was 70 % after 24 h and 90 % after 48 h. At concentrations of 0.05 mg l^{-1} 100 % mortality was observed.

Atrazine had no effect on *Daphnia* in the concentration range 0.005 to 10 mg l^{-1} (Figure 7.4). The affect of the organophosphate gusathion is shown in Figure 7.5 and the concentration range tested was 1 x 10^{-5} to 200 mg l^{-1} gusathion. At low concentrations (1 x 10^{-5} to 1 x 10^{-3}) the *Daphnia* were not effected.

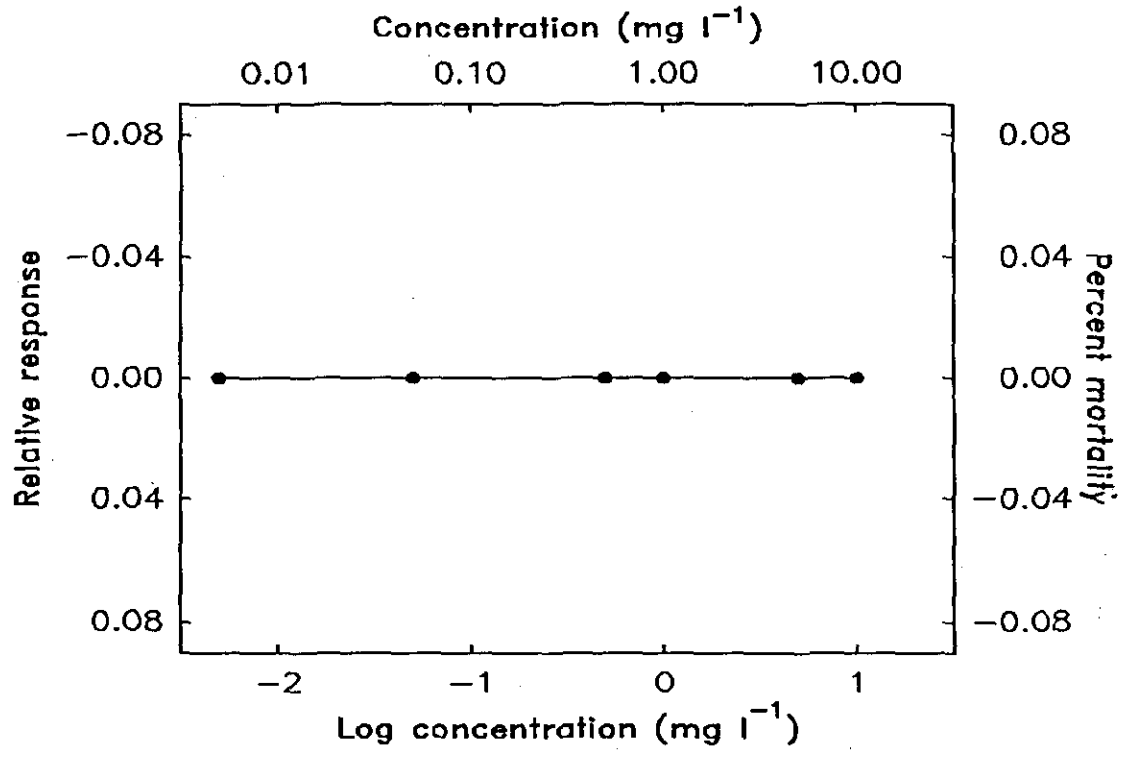


Fig 7.4. Percent mortality against different atrazine concentrations using *Daphnia pulex* as test organism; where (○) = 24 h and (●) = 48 h.

After 24 h exposure to a concentration of 0.01 mg l^{-1} 70 % of the organisms were dead and after 48 h it rose to 90 %. Hundred percent mortality was observed at concentrations ≥ 0.1 mg l^{-1} gusathion.

The response of *Daphnia* towards phenol toxicity indicated a sigmoidal response curve (Figure 7.6). No affect was seen at concentrations of 0.01 to 1 mg l^{-1} phenol when exposed for 24 h. However, after 48 h exposure to a concentration of 1 mg l^{-1} 30 % of the organisms were killed. A concentration of 10 mg l^{-1} phenol resulted in a 90 % mortality after 24 h and 100 % after 48 h. Only after 24 h exposure to a phenol concentration of 100 mg l^{-1} was 100 % mortality observed.

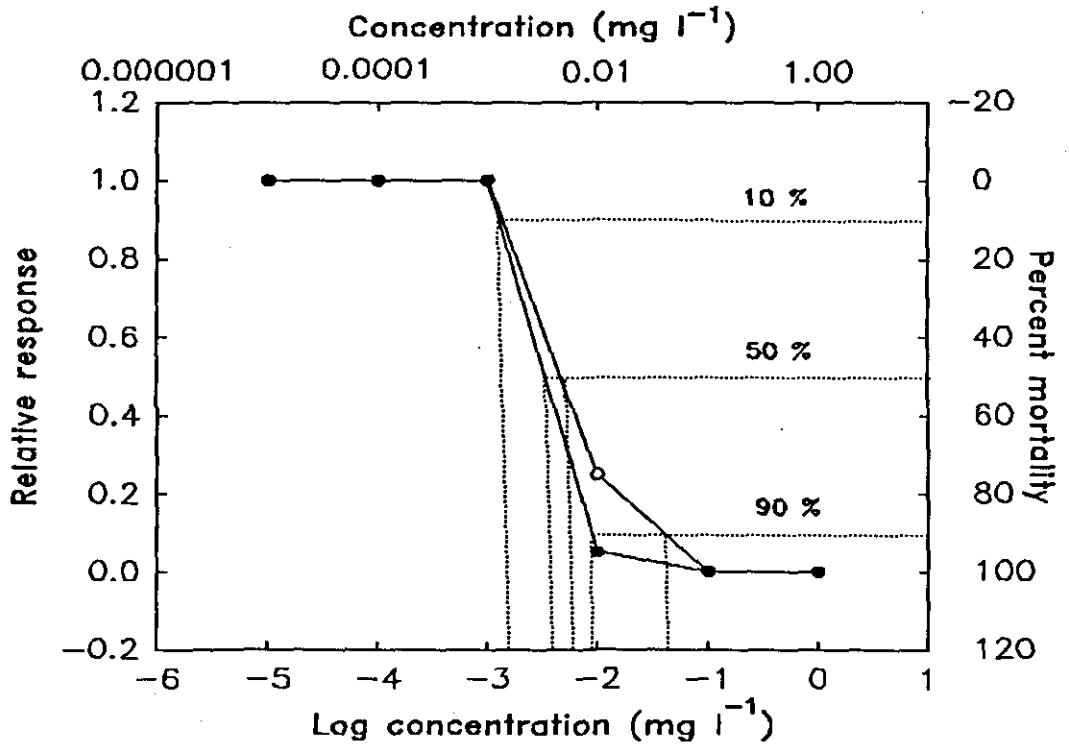


Fig 7.5. Percent mortality against different gusathion concentrations using *Daphnia pulex* as test organism; where (○) = 24 h and (●) = 48 h.

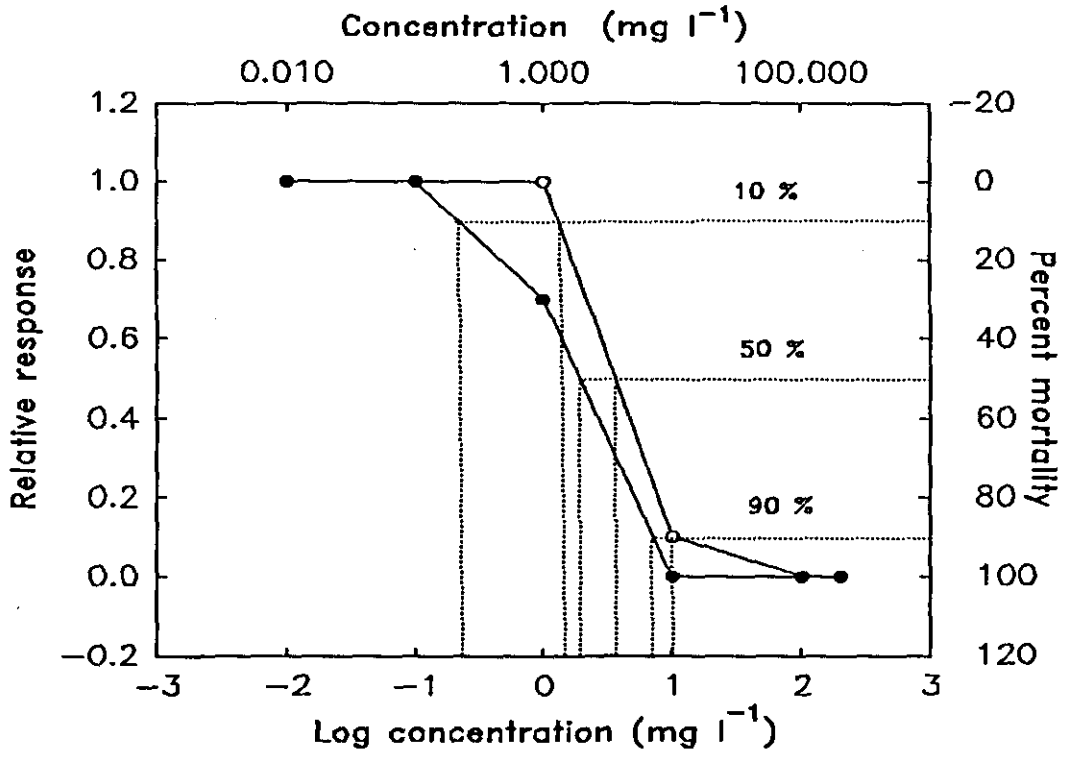


Fig 7.6. Percent mortality against different phenol concentrations using *Daphnia pulex* as test organism; where (○) = 24 h and (●) = 48 h.

Tertiary treated municipal sewage at 25, 50, 75 and 100 % had no effect on *Daphnia*.

Table 7.2. Summary of results from toxicity tests with *Daphnia pulex* (LC values in mg l^{-1}). *nr = no response

Compound	24 h			48 h		
	LC10	LC50	LC90	LC10	LC50	LC90
Cadmium	0.110	0.159	0.229	0.100	0.149	0.229
Copper	0.011	0.015	0.060	0.011	0.014	0.020
Mercury	0.006	0.009	0.037	0.005	0.008	0.020
Atrazine	*nr	nr	nr	nr	nr	nr
Gusathion	0.001	0.005	0.039	0.001	0.003	0.009
Phenol	1.259	3.631	10.00	0.200	1.962	6.761

Discussion

Daphnia appears to be very sensitive to copper and mercury with LC50 values as low as 11 µg l^{-1} and 9 µg l^{-1}, respectively, in comparison to cadmium with a LC50 of 160 µg l^{-1}. This indicates about a 10 times greater tolerance towards cadmium toxicity. Biesinger and Christensen (1972) measured the toxicity of cadmium to *Daphnia magna* during an entire life cycle and it was found that 50 % of the daphnids exposed to cadmium concentrations of 5 µg l^{-1} were killed in three weeks. However, several invertebrate species have been found to be much less sensitive to cadmium when subjected to the acute tests as sited above (US EPA, 1976). The cadmium LC50 value of 169 µg l^{-1} can easily detect the effluent standard of 0.05 mg l^{-1} as laid down by law (Table 2.2, Chapter 2) for discharge into freshwater systems.

The effluent standards laid down for copper and mercury are 1 and 0.02 mg l^{-1} respectively. The LC50 values of 0.015 and 0.009 mg l^{-1} as determined in this study, respectively, also indicate that the *Daphnia* test would be suitable to use as a screening toxicity method. In fact the *Daphnia* test is extremely sensitive to copper with a LC50 value 66 times below the effluent standard. It should, however, be remembered that an effluent, when dumped into a waterbody will be diluted to some extent and this should also be taken into consideration. The concentration of copper in freshwater exhibiting no harmful effects to several aquatic species is about 5 to 15 µg l^{-1} according to the US EPA (1976) which corresponds favourably with our LC50 value of 15 µg l^{-1}.

Gusathion which is used as a pesticide is very toxic to *Daphnia* with a LC50 of 3 µg l^{-1} after 48 h. This is well within the 0.01 µg l^{-1} threshold for freshwater and marine aquatic life (Table 2.2, Chapter 2). Phenol, although being a volatile substance, showed increasing toxicity with time of exposure where the LC50 values were 3.6 mg l^{-1} after 24 h and 2 mg l^{-1} after 48 h. It has been shown that phenol has 24 hour LC50's of 5 mg l^{-1} for trout embryos (Albersmeyer and von Erichsen, 1959) which is slightly more than the LC50 values determined in this study. In the sewage sample various daphnids as well as some copepods were identified which confirm that this effluent water was not toxic to zooplankton.

From the observations discussed above, water quality limitations based on toxicity data for daphnids seems to be biased towards certain substances only. The inability of *Daphnia* to detect the herbicide atrazine, which severely influences aquatic plants, proves this point. Despite this shortcoming the method proved to be reliable and repeatable.

CHAPTER 8

COMPARISON OF ALGAL AND ZOOPLANKTON ASSAY PROCEDURES FOR DETECTING TOXIC SUBSTANCES IN NATURAL WATERS

Introduction

The transport and fate of potentially hazardous chemicals in the natural environment have received increasing attention in recent years, necessitating the development of bioassay procedures. As primary producers in aquatic ecosystems, algae are important test organisms for assessing the impact of chemical substances on water quality. Efforts by various laboratories have resulted in the development of the "bottle test" (US EPA, 1971). This reliable and reproducible test is widely used for determining the algal growth potential of natural waters. This procedure has also been used in metal toxicity testing (Miller *et al.*, 1978), and for determining nutrient limitations (Turbak *et al.*, 1986). The bottle test requires 2 - 3 weeks to complete (Miller *et al.*, 1978) and is, therefore, not suitable as a rapid screening test. The International Standards Organization (ISO) and the Organization for Economic Cooperation and Development (OECD), however, accepted an algal bioassay test with a duration of 72 h (Nyholm and Källqvist, 1989).

Photosynthesis is a rapid reaction and the response to factors that inhibit this process are observed within seconds (Cullen and Lewis, 1988). Progress in understanding phytoplankton photosynthetic dynamics is to a large extent due to three techniques, namely the ^{14}C-method for measuring primary productivity (Steemann Nielsen, 1952), the fluorometric method of measuring chlorophyll *a* (Holm-Hansen *et al.*, 1965) and the oxygen evolution method to measure photosynthetic rates and efficiency (Dubinsky *et al.*, 1987).

Radiocarbon uptake (Steemann Nielsen, 1952) is a well known method in aquatic productivity estimations and pollution studies. Lewis and Smith (1983) have developed a small volume, short-incubation-time method for the determination of algal photosynthetic rates as a function of incident irradiance. This method has proven to be useful, both in the laboratory and in the field, for analysis of short time scale responses of algae to changes in available light. This method could prove to be useful as a bioassay technique for the determination of the response of algae to toxic substances.

It is essential when doing bioassays, that the experimental conditions should be reproducible. This implies precise control of the experimental conditions such as temperature, irradiance and the assessment of either ^{14}C-uptake or O_2-evolution. Clark-type O_2 electrodes offer advantages over, for example the ^{14}C-uptake method in that oxygen production or consumption could be measured continuously (Dubinsky *et al.*, 1987).

The fluorescence from *in vivo* chlorophyll *a* has been used as a convenient index for chlorophyll *a* determination in limnological studies. Fluorescence at room temperature is mainly from the chlorophyll *a* of photosystem II (PSII) and the yield is primarily determined by the electron transfer in and around PSII. The yield has been known to depend on the redox state of the primary electron acceptor (Q) of PSII. The yield is high when Q is reduced (closed state), and becomes low (open state) when Q is oxidized. Q has been identified as a plastoquinone bound to the protein complex of PSII (van Gorkom, 1974).

The herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), inhibits the reoxidation of Q and after addition results in maximum fluorescence. According to Roy and Legendre (1979) this gives a more quantitative measure of the chlorophyll *a* concentration. The enhancement of fluorescence by DCMU reflects photosynthetic electron flow (Ishimaru *et al.*, 1985).

Table 8.1. Results from toxicity tests with *Selenastrum capricornutum* and *Daphnia pulex*. EC and LC values in mg l^{-1}. AGP = Algal growth potential, IVF = *in vivo* chlorophyll *a* fluorescence, 14C = ^{14}C-uptake and O2 = O_2-evolution with *Selenastrum capricornutum* and DP = Zooplankton mortality tests with *Daphnia pulex*. *nr = no response.

Compound	AGP			IVF			14C			O2			DP		
	EC10	EC50	EC90	EC10	EC50	EC90	EC10	EC50	EC90	EC10	EC50	EC90	LC10	LC50	LC90
Copper	0.151	0.227	0.290	*nr	nr	nr	0.246	0.736	nr	0.280	nr	nr	0.011	0.014	0.020
Cadmium	0.053	0.101	0.136	nr	nr	nr	0.009	0.077	nr	0.100	nr	nr	0.100	0.148	0.229
Mercury	0.005	0.030	0.076	nr	nr	nr	0.049	0.061	0.085	0.021	nr	nr	0.005	0.009	0.020
Atrazine	0.375	0.784	1.715	0.146	0.335	1.900	0.013	0.049	0.176	0.031	0.222	0.751	nr	nr	nr
Gusathion	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	0.001	0.003	0.009
Phenol	3.003	nr	nr	11.22	145.1	nr	4.728	nr	nr	0.760	nr	nr	0.200	1.962	6.761

As for algal bioassays, aquatic animals have also been used as test organisms in toxicity tests. Microbial toxicity tests, including algal tests, differ from toxicological assays with animals because the former usually involve a very large number of organisms that can be viewed as a continuum with certain properties. Toxicity tests with animals are carried out with few test subjects and the response is often quantitative or categorical (e.g. death) whereas the response obtained in most microbial toxicity tests is a quantitative continuous variable (Nyholm *et al*, 1992). The water flea *Daphnia*, has been used in freshwater toxicity studies for over a century and results obtained by different investigators are comparable.

In this chapter the results obtained with the different assay methods are compared. The purpose was to determine whether;
1. there is agreement between the methods,
2. which methods are the most sensitive, and
3. which compounds are best suited for detection by the different methods.

A summary of the results obtained with the different methods and compounds tested in this study, is given in Table 8.1. From this it can be seen that;

1. The heavy metals Cu, Cd and Hg had no effect over the short term on algal *in vivo* chlorophyll *a* fluorescence.
2. Cu, Cd and Hg over the concentration range tested, inhibited O_2-liberation with only 10%.
3. The ^{14}C-uptake of *Selenastrum* was inhibited with 50 % by Cu, Cd and Hg.
4. Cu, Cd and Hg inhibited algal growth over the long term with 90 % in the AGP tests.
5. 90 % mortality was caused by the heavy metals in the zooplankton mortality assays.
6. Atrazine caused a 90 % inhibition of algal response, irrespective of the algal method used.
7. Atrazine had no effect on *Daphnia pulex*.
8. Gusathion had no effect on the algae, but greatly affected *Daphnia*.
9. Phenol caused little algal inhibition, the most sensitive algal test was the O_2-liberation measurements using *Selenastrum* as test organism.
10. The *Daphnia* test was the most sensitive to phenol toxicity.

Table 8.2. The order of sensitivity of the different methods in terms of the compounds tested. AGP = Algal growth potential, IVF = *in vivo* chlorophyll *a* fluorescence, 14C = ^{14}C-uptake, O2 = O_2-evolution measurements of *Selenastrum capricornutum* and DP = zooplankton lethality tests with *Daphnia pulex*. EC = Effective concentration and LC = lethal concentration to inhibit or kill a certain percentage of test organism performance.

Compound	EC\LC	Bioassay sensitivity order
Copper	10	DP > AGP > 14C > O2
Cadmium		14C > O2 = DP > AGP
Mercury		AGP = DP > O2 > 14C
Atrazine		14C > O2 > IVF > AGP
Phenol		O2 > DP > AGP > 14C > IVF
Copper	50	DP > AGP > 14C
Cadmium		14C > AGP > DP
Mercury		DP > AGP > 14C
Atrazine		14C > O2 > IVF > AGP
Phenol		DP > IVF
Copper	90	DP > AGP
Cadmium		AGP > DP
Mercury		DP > AGP > 14C
Atrazine		14C > O2 > AGP > IVF

Table 8.3. Relative sensitivity of the algal growth potential (AGP) test, *in vivo* fluorescence (IVF), ^{14}C-uptake (14C), O_2-evolution (O2) toxicity tests with *Selenastrum capricornutum* and zooplankton toxicity test (DP) with *Daphnia pulex* expressed as the ratio of ECs and LCs. A ratio = 1 indicates no difference, > 1 indicates a greater sensitivity of the divisor, and <1 a greater sensitivity of the dividend.

Compound	EC\LC	AGP/DP	IVF/DP	14C/DP	O2/DP	AGP/IVF	AGP/14C	AGP/O2	IVF/14C	IVF/O2	O2/14C
Copper	10	13.73	0.00	22.00	26.00	0.00	0.61	0.54	0.00	0.00	1.14
Cadmium		0.53	0.00	0.09	1.00	0.00	5.89	0.53	0.00	0.00	11.11
Mercury		1.00	0.00	10.00	4.20	0.00	1.00	0.22	0.00	0.00	0.43
Atrazine		0.00	0.00	0.00	0.00	2.57	28.85	12.10	11.23	4.71	2.38
Gusathion		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phenol		15.00	56.00	24.00	3.80	0.27	0.64	3.95	2.37	14.76	0.16
Copper	50	16.20	0.00	53.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00
Cadmium		0.68	0.00	0.53	0.00	0.00	1.31	0.00	0.00	0.00	0.00
Mercury		3.00	0.00	7.00	0.30	0.00	0.49	0.00	0.00	0.00	0.00
Atrazine		0.00	0.00	0.00	0.00	2.34	16.00	3.53	6.84	1.51	4.53
Gusathion		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phenol		0.00	71.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Copper	90	14.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cadmium		0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mercury		3.80	0.00	4.20	0.00	0.00	0.90	0.00	0.00	0.00	0.00
Atrazine		0.00	0.00	0.00	0.00	0.90	9.74	2.28	10.80	2.53	4.27
Gusathion		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phenol		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Shown, in Table 8.2, is a comparison between the sensitivity order of the five methods (AGP, *in vivo* fluorescence, ^{14}C-uptake, O_2-evolution and *Daphnia* mortality) to various compounds (copper, cadmium, mercury, atrazine, gusathion and phenol) at EC10, EC50 and EC90 levels of response. From this and the results shown in Table 8.1 it is clear that not only did the sensitivity between the methods used differ, but also between the compounds tested, depending on whether a 10 %, 50 % or 90 % response was measured. From the results shown in Table 8.2 it is clear that the *Daphnia* mortality and the ^{14}C-uptake tests, were the most sensitive at the various EC/LC concentrations. This was followed by the algal growth potential test (AGP).

The largest difference in sensitivity was between *Daphnia* mortality and *Selenastrum in vivo* chlorophyll *a* fluorescence when the response to phenol at 50 % inhibition/mortality were measured. The former was 70 times more sensitive than the latter (Table 8.3). What is of further interest concerning the results shown in Table 8.3 is to note the decreasing sensitivity of the short term methods to detecting toxic substances with an increase in EC/LC concentrations. Only on a few occasions could the short term methods detect EC90 concentrations. The two exceptions were atrazine and gusathion, but it should be noted that atrazine did not affect the *Daphnia* and gusathion did not affect the algae.

From a comparison between toxicity of the different toxic substances, as measured with the different methods, it is obvious that gusathion was the most toxic to *Daphnia* with an LC50 of 3 $\mu g\ l^{-1}$. This compound had no effect on the algae and of the compounds that affected both the algae and *Daphnia*, mercury was the most toxic. The LC50 concentration for the *Daphnia* test was 9 $\mu g\ l^{-1}$ which is 3 times less than that measured by the most sensitive algal test, being 27 $\mu g\ l^{-1}$ with the algal growth potential. Considering only the short term ($\leq$ 4 h) tests, i.e. acute toxicity tests and screening bioassays, atrazine affected ^{14}C-uptake after only 30 min the most with an EC50 of 50 $\mu g\ l^{-1}$. The acute short term ^{14}C-uptake test were much more sensitive to atrazine toxicity than long term (72 h) algal growth potential tests.

Discussion

The results clearly illustrate that there are large variations between the different toxicity methods used and their responses to toxic compounds. Different compounds not only affect different organisms differently (e.g. in the trophic chain), but depending on the test method used, compounds affect the same organism differently. Differences between different organisms in the trophic chain could be expected, whereas in this study plants (algae) and animals (*Daphnia*) were used as test organisms. The fact that the algae did not react to gusathion and the *Daphnia* not to atrazine, clearly emphasise this point. Differences in the reaction to toxic substances by the same organism (in this case *Selenastrum*), could be attributed to the metabolic role which the specific compounds have.

Algae may be the most suitable test organisms for toxicity studies, because they have almost all the metabolic pathways which living organisms have. Our tests did not exhaust all these possibilities, where tests could be designed to target, e.g. photosynthesis alone. This in itself could be subdivided into tests dealing only with carbon uptake (e.g. the ^{14}C-method); enzymes important in carbon fixation such as Rubisco; oxygen liberation or uptake; electron transport in the photosystems (e.g. *in vivo* chlorophyll *a* fluorescence); general growth (e.g. the AGP test); cell division; pigment ratio's especially chlorophyll *a/b* and chlorophyll *a*/carotenoids; the accumulation of certain products during stress; or respiratory electron transport measurements (ETS). Oxygen liberation or uptake measurements would allow for a distinction to be made between gross and net photosynthesis, as well as dark respiration. Thus two completely different metabolic pathways are used in these measurements. From the above it is clear that much can still be done with regards to the uses of algae as test organisms in bioassays.

The differences found between chronic (long term) assays and acute (short term or screening) assays could be expected but these differences were variable which makes the responses unpredictable. Hersh and Crumpton (1987) suggested that effects caused by toxins must be determined when nutrient and toxin concentrations are less affected by cell

number. This is best accomplished early in the experiment. Atrazine has a short term (or algistatic) effect on algal photosynthesis as illustrated by the higher sensitivity in comparison to algal growth potential measurements. This observation has also been made by Abou-Waly *et al.*(1991) who noted that the algal cells recovered their normal vitality after 3 days exposure. It was also found that the toxicity of Cu^{2+} decreased when the concentration of cells in the test suspension increases (Barashkov and Kiristayeva, 1977), which is confirmed by our EC10 results (Table 8.1).

Conclusions

Algal and zooplankton assays of the heavy metals; copper, cadmium and mercury, the organochloride (atrazine), the organophosphate (gusathion), phenol, and treated municipal sewage effluent were performed using *Selenastrum capricornutum* and *Daphnia pulex*. The test methods used were; algal growth potential (AGP); short-term (0 - 4 h) *in vivo* chlorophyll *a* fluorescence; ^{14}C-uptake; O_2-evolution of the algae and mortality of *Daphnia*. The results clearly showed that;

1. *Selenastrum capricornutum* is a suitable test alga because the cells grew well in both batch and semi-continuous cultures; responded well to photosynthetic inhibition by toxins; and chlorophyll *a* could easily be extracted for growth measurements.
2. *Daphnia pulex* is also a suitable organism for toxicity testing and can easily be maintained in the laboratory.
3. The use of a standardized methodology (testing protocols) for toxicity measurements is crucial for repetitive measurements and comparison purposes.
4. Algae are suitable for both acute and chronic toxicity testing, where the ^{14}C-uptake method gave an indication of the water quality within 30 minutes. Screening bioassays should also be combined with longer term performance.
5. Different compounds influence different organisms differently, especially when they are from different levels in the trophic chain.
6. By targeting different metabolic pathways or processes in the same organism, different compounds gave different sensitivities in terms of toxicity.
7. That various options are open for further investigations using algae and that they may prove to be the ideal toxicity test organisms.

Recommendations

Based upon the results the following recommendations are made;

1. The ^{14}C-uptake method, because of its rapid response, should be used for screening purposes. Incubating at I_k irradiancies in the Photosynthetron allows for many samples to be processed at the same time, while working with small volumes of algal material.
2. *In vivo* chlorophyll *a* fluorescence showed potential as a means of testing for toxic substances and needs to be investigated further.
3. Further research should be carried out on algae to investigate various metabolic pathways not yet included in toxicity measurements and other indicators of the physiological state of the test organisms.
4. That more than one level in the trophic chain be used when investigating potentially toxic compounds or environmental quality.
5. That experimental protocols be standardized and if possible simplified to restrict experimental variations and error.

CHAPTER 9

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