

Field bioassays for side-effects of pesticides: final report

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FIELD BIOASSAYS FOR SIDE-EFFECTS OF PESTICIDES

FIELD BIOASSAYS FOR SIDE-EFFECTS OF PESTICIDES

- FINAL REPORT -

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CML report 112 – Section Ecosystems and Environmental Quality

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FOREWORD AND ACKNOWLEDGMENTS

This report presents the results of a four-year field study aimed at developing field trials for the side-effects of pesticides. Annual progress reports are available in Dutch. A progress report of the first two years is available in English as well.

In the course of this study we have received assistance from many sides. We extend our particular thanks to the members of the advisory committee: H. van der Baan, M. van der Gaag, J.A. van Haasteren, and J. Prast (all Ministry of Housing, Physical Planning and Environment), C. van de Guchte (National Institute of Inland Water Management), J.H. Koeman (Dept. of Toxicology, Wageningen Agricultural University), R. Luttik (National Institute of Public Health and Environmental Protection), R. Rondaij (Staring Centre), H.J.M. Straathof (National Plant Protection Service), A.J. Termorshuizen (Dept. of Phytopathology, Wageningen Agricultural University) and H.A. Udo de Haes (Centre of Environmental Science, Leiden University).

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From several institutes we received test organisms (sometimes to start our own culturing programme): sticklebacks from the department of Ethology of Leiden University, water fleas from RIVM, *Chironomus* midge eggs from TNO-Delft, snails from the faculty of Biology of Free University Amsterdam, caterpillars from the Dutch Butterfly Foundation and from the department of Entomology of Wageningen Agricultural University, and homozygotic oilseed seeds from Van der Have Research.

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We extend our special thanks to the students, analysts and colleagues who carried out parts of the project and sometimes spent long days doing routine work: Evert Heemskerk, René van Kooy, Margriet van der Nagel, Frode Numan, Hiddo Rombout, Nellie Sinnige, Sander Versprille, Ronald Walgreen and Paul de Wit. Last but not least, we would like to thank Kees Canters, who acted as the head of this project in the first year.

Frank M.W. de Jong
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Leiden, December 1994

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SUMMARY

Since 1986 CML has been conducting a series of desk studies on the side-effects of pesticides on non-target organisms, commissioned by the Dutch environment ministry. One of the main conclusions of these studies was that there is a lack of pertinent field research, despite the fact that side-effects are to be expected. Therefore, as a follow-up to these desk studies, in 1991 the environment ministry commissioned CML to conduct a four-year field study to investigate the perspectives for field trials. The aim of this study is:

> **to design, validate and standardize field trials for evaluating direct and indirect side-effects of pesticides in the aquatic and the terrestrial environment.**

In 1991 an experimental field research programme was started, in which bioassay methods were used as the most controlled way of conducting a field study. Selection of test species included their representativity for different important ecological functions of the ecosystem: primary producers, herbivores, carnivores and decomposers. By using these selection criteria the tests can give indications of side-effects on fundamental ecological processes. In this way the test results form a basis for conclusions on effects on the "General Environmental Quality".

This report present the results of the field bioassays and the proposed guidelines for conducting the bioassays. Furthermore, the interpretation of the results is discussed. Separately from this report, in a second report (CML report 117) field trials will be discussed at a more general level; different types of field trials will be discussed and compared, and a framework for conducting field trials will be designed.

Aquatic bioassays

In the first and second year of this study the use of field bioassay methods was tested, employing a variety of organisms with different ecological functions. Tests were carried out in and near treated agricultural parcels in a variety of crops and with a number of pesticides. The results were compared with results from untreated plots. Test species included periphyton algae and duckweeds *Lemna minor*, *L. gibba*, and *Spirodela polyrhiza* (primary producers), water fleas *Daphnia magna* and water snails *Lymnaea stagnalis* (herbivores), sticklebacks *Gasterosteus aculeatus* and larvae of phantom midges *Chaoborus* (carnivores), and larvae of midges *Chironomus riparius* (herbivores/decomposers), isopods *Asellus*, amphipods *Gammarus*, and decomposition of American River Weed *Elodea* spp. The results were found to vary.

In the third and fourth year of the study tests with the most suitable species were developed further under more controlled conditions. These test organisms included periphyton algae, duckweeds, larvae of midges *Chaoborus* and amphipods *Gammarus*. In the laboratory, a pilot experiment with litterbags was also carried out. The results of these studies are summarized below.

Periphyton algae

Experiments were conducted in the laboratory and in enclosures using the herbicide diquat. In contrast with the *in situ* results, no effects on chlorophyll-a levels were found in the laboratory (20 μg/l diquat) or in enclosures (270 μg/l diquat). Thus, no clear indications were obtained that the bioassay in its present form is useful for evaluating pesticide side-effects. Subject to the possibility that for other compounds better results might be achieved, at this point it is concluded that this bioassay is not useful for evaluating the side-effects of herbicides *in situ*.

Duckweeds

Experiments with duckweeds were carried out once in containers on an experimental field and twice in enclosures in a polder ditch using the herbicide diquat. The results of the container study show that *Spirodela polyrhiza* and *Lemna gibba* are highly sensitive to diquat: growth inhibition was measured at a deposition below 0.2% of the sprayed (recommended) field rate; for *L. minor* no growth inhibition was found at this rate.

The results of the two enclosure experiments show that of all tested species *S. polyrhiza* was the most sensitive to diquat, showing complete mortality at 7% of the recommended field rate, and severe damage and growth inhibition for at least four weeks at 0.7% of the recommended field rate. *L. gibba* exhibited an intermediary sensitivity, being severely damaged at 7%. In the third week there a complete recovery of growth. At 0.7% of the recommended field rate there was little mortality. *L. minor* was the least sensitive to diquat, showing no mortality and growth recovery in the second week at 7% of the recommended field rate. The bioassay does not require costly technical provisions and results are obtained within two weeks of application. For this species a guideline is proposed.

Larvae of midges

The bioassay was developed using third- and fourth-instar larvae of the phantom midge *Chaoborus crystallinus* (De Geer). In the laboratory dose-effect experiments were carried out with the insecticides diflubenzuron and methyl-parathion. Diflubenzuron primarily inhibited moulting; at seven days, 50% reduced emergence was calculated to be 0.95 μg/l. Mortality from incomplete moults was also observed. Methyl-parathion caused immobilization and mortality, and the 7-day EC_{50} and LC_{50} were calculated to be 0.51 and 1.10 μg/l, respectively.

The bioassay was tested in a semi-field situation. Four enclosures within a drainage ditch were stocked with exposure chambers containing *C. crystallinus* larvae, and were sprayed with methyl-parathion. This experiment was performed twice. Effects differed widely between the experiments, resulting in a calculated 7-day LC_{50} of 49.6 μg/l in the first experiment, and < 1.07 μg/l in the second. Immobilization was almost negligible and was seen only in the second experiment at two days post-treatment. Differences in effects between the two enclosure experiments seemed to be caused mainly by differences in levels of free methyl-parathion. In the second enclosure experiment water samples were taken to the laboratory and toxicity to *C. crystallinus* was assessed. Effects in the laboratory were much lower than in the enclosures. It therefore seems that laboratory toxicity tests may not be representative of effects in the field. From these experiment it is

concluded that the bioassay with *C. crystallinus* is suitable to investigate effects from a single insecticide application.

Bioassays were also carried out in twelve replicated laboratory model ecosystems, contaminated continuously with the insecticide chlorpyrifos at 0.1 μg/l, or the herbicide atrazine at 5 μg/l. Increased mortality was found in the chlorpyrifos-treated systems within two weeks, indicating that the bioassay is also potentially suitable for investigating effects of exposure to low insecticide levels during prolonged periods of time.

Finally, the practical use of the field bioassay in real-field situations was tested, selecting uncontaminated drainage ditches, varying in width and soil type of the beds. Survival was high for most ditches, suggesting that the method is widely applicable. For this species a guideline is proposed.

Amphipods

The field bioassay was tested in four enclosures within a drainage ditch. The enclosures were stocked with exposure chambers containing juvenile *Gammarus* spp., and were sprayed with methyl-parathion. This experiment was performed twice. In the second experiment effects were stronger than in the first, resulting in 50% immobilization at 5.59 μg/l in the first, and 1.56 μg/l in the second experiment. In the second experiment, however, mortality in the untreated group was also relatively high (18.3%), which can be ascribed to the use of very small (young) amphipods in the second experiment. Differences in effects between the two enclosure experiments seemed to be caused mainly by differences in levels of free methyl-parathion.

Also, the practical use of the field bioassay in real-field situations was tested, selecting uncontaminated drainage ditches, varying in width and soil type of the beds. Survival was high for ditches with sandy and clay beds; for ditches with peaty beds the survival was variable, but this was probably caused by a blanket of duckweed resulting in low oxygen levels. The results from this experiment suggest that the method is widely applicable in ditches that are not completely overgrown by duckweed. For this species also a guideline is proposed.

Litterbags

In the aquatic environment a pilot experiment with litterbags, as used in the terrestrial studies, was carried out in the laboratory. After seven days inhibition of decomposition was seen in the captan-treated group; after eleven days no differences in decomposition were found. These results indicate that the litterbag method is also potentially suitable for aquatic decomposition studies.

Terrestrial bioassays

In the terrestrial environment tests were carried out using oilseed rape *Brassica napus* and *Poa annua* (primary producers), caterpillars of the large white butterfly *Pieris brassicae* (herbivores), and litterbags with rape *Brassica oleracea* leaf discs (representing decomposition). In 1992 the bioassays were tested in maize, potato and fruit parcels, and in a tree nursery. In 1993 and 1994 the bioassays were developed under the more controlled

conditions of an experimental field. Bioassay units were placed in and up to 16 m downwind from an experimentally sprayed plot. Deposition of the spray drift was measured using water-sensitive paper.

Plants

The results of the bioassays with *Brassica napus* and *Poa annua* showed significant differences in sensitivity between the dicotyledonous and monocotyledonous species, depending on the compound used. Effects were found at extremely low deposition rates; sometimes effects were found below the detection limit of the method with the water-sensitive paper. At the highest wind speeds occurring in the experiments (5 m/s) deposition was found at 16 m downwind from the treated plot. Differences of 10% between means could be estimated with $P<0.05$.

The results could be validated in 1994. At moderate wind speeds $Edist_{50}$ for plant growth was approximately five metres from the treated plot for the tree compounds tested (glyphosate, diquat and bentazone). Replicated experiments showed relatively high variations in ED_{50} values. This was probably due to variations in environmental conditions, during cultivation, during exposition, or in the period after exposition.

The bioassay is suitable for assessing the effects of drift-diminishing measures, e.g. buffer zones, or for estimating a desired buffer zone width. Furthermore, the bioassay can be used for the detection of low deposition rates. For use in the procedure for authorization, the method must be conducted following exact procedures, as stated in the proposed guideline.

Caterpillars

For the field bioassay for side-effects of insecticides larvae of the large white butterfly *Pieris brassicae* were used. Firstly, indoor experiments were conducted using diflubenzuron and pirimicarb. Larvae, 48-72 hours of age, were placed on host plants *Brassica napus* and *Brassica oleracea* and exposed to a range of dilutions of the compounds (100%, 10%, 2%, 1% and 0% of the actual commercial application rate: diflubenzuron: 10000 $\mu g/m^2$; pirimicarb: 25000 $\mu g/m^2$). Effects on survival were found for both compounds from 2% of the actual application rate upwards.

In two field trials with diflubenzuron, bioassay units were placed up to 16 m downwind from a treated plot. The wind speed at the time of spraying was 3.5 m/s and 4.5 m/s, respectively. ED_{50} values for deposition as low as 13 and 3 $\mu g/m^2$ were found. The results show that, compared to the indoor experiments, in the field experiment effects on larvae of *P. brassicae* were found at lower exposure rates.

This relatively simple bioassay appeared to be very suitable for tracing the side-effects of low deposition rates of an insecticide in the field. It can therefore serve as a useful tool for estimating the effectiveness of buffer zones, etc.

Decomposition

A major effort was made to optimize a method with litterbags. In the most optimal situation the side-effects of fungicides on decomposition of organic matter were investigated using litterbags of nylon (25 μm mesh) measuring 20 x 20 cm. 20 leaf discs of

Chinese cabbage *Brassica oleracea*, dried for three hours at 100°C, were placed in the litterbags. The litterbags were placed in the field and covered with 1 cm of a standard soil (sand). Shortly after placement, the litterbags were exposed. After one week the dry weight of the leaf discs was determined. Litterbags were placed in and downwind from a treated plot, and results were compared to those from an untreated plot. Tests were conducted in field crops of fruit and potatoes, in a glasshouse and at an experimental plot.

In agricultural practice negative effects on decomposition were found after application of two fungicides (captan and maneb) up to 10 m from the treated plot in the field situation. In the range of 0 to 20 m from the treated plot, the decomposition rate (70% to 85% dry weight loss) was significantly positively correlated (captan: $R=0.57$, maneb: $R=0.56$, $P<0.01$) with the distance from the treated plot. In one case the captan content of the soil was measured and a significant negative correlation was found between captan concentration and decomposition ($R=-0.62$, $P<0.01$).

In the glasshouse experiments these results could be validated. Effects on decomposition were small ($\pm$ 10%) but significant and were found in several experiments. On the experimental field plot effects could not be validated. In a preliminary experiment aimed at soil fungi a relation between exposure to captan and the number of soil fungi in agar was found. From the results it is concluded that the use of litterbags has potential, but is not yet suitable for use in a standard field trial.

Conclusions

After four years of field study it can be concluded that the use of bioassays is a relatively simple method for tracing the direct side-effects of pesticides. The bioassays are not suitable for tracing indirect effects. The direct effects could be traced at low exposure rates outside the target area at relatively low cost.

The bioassays appear to be useful for estimating the effectiveness of buffer zones and can be used as a tool for the determination of drift at low deposition rates.

1 INTRODUCTION

1.1 Background and motivation

In the Netherlands, annual agricultural pesticide use stands at about 20-21 x 10^6 kg active ingredient (a.i.) (MJP-G, 1991). amounting to an average of 14 kg/ha/y. Although the compounds are used within the agricultural target areas, a fraction disperses to the surrounding environment[1]. Both within the treated plots and in the surrounding area the pesticides can contact non-target organisms, and side-effects (negative effects on non-target organisms) are therefore extremely likely.

Two types of side-effects (Fig. 1.1) can be distinguished (*cf.* De Snoo *et al.*, 1994). First, there are the direct side-effects resulting from a substance's toxicity to an organism. These effects may be either primary or secondary. Primary poisoning occurs when the active ingredient has a deleterious impact not only on target organisms but also on non-target organisms. Secondary poisoning occurs at a higher trophic level, with lower-level organisms acting as intermediaries. This type of effect occurs mainly with persistent pesticides. Second, there are indirect side-effects: non-toxic effects on species of concern following, *inter alia*, from changes in the food chain (e.g. disappearance of a prey species) or changes in habitat (e.g. disappearance of vegetation).

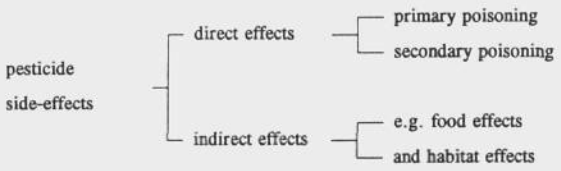


Figure 1.1 Categorization of pesticide side-effects.

Since 1986 CML has been studying these side-effects, commissioned by the Dutch environment ministry. In a series of desk studies, side-effects on vertebrates (De Snoo & Canters, 1990), invertebrates and aquatic fauna (Canters *et al.*, 1990) and fungi and vascular plants (De Jong *et al.*, 1992) have been investigated.

The main result of these studies is that, despite the legislative procedure in force, side-effects are to be expected. At present many standard laboratory tests are available; in the Netherlands the overwhelming emphasis is on mesocosm studies, with hardly any field research being carried out. This lack of field research and uncertainties concerning extrapolation of results from the laboratory to the field have led to proposals for designing field trials (De Jong *et al.*, 1990) based on desk studies. The uncertainties regarding

[1] In arable crops this fraction may be up to 25% and in glasshouse areas up to 55% (MJP-G, 1991); a major proportion (80%-90%) of this quantity enters the atmosphere as a result of drift or volatilization. For certain compounds an even greater fraction disperses: in the case of soil disinfectants the fraction entering the atmosphere this may be as high as 50%-80% of the applied dosage (MJP-G, 1991; IMP-M 1987-1991, 1986).

extrapolation concern dispersal and exposure as well as the differences in sensitivity between individuals and species. In addition, other stress parameters may influence sensitivity in the field. As a follow-up to these desk studies, in 1991 the environment ministry commissioned CML to conduct a four-year field study to investigate the scope for field trials.

In the first year, 1991, the field study started with bioassays in the aquatic environment, aimed at charting the side-effects in the field outside the target area. A variety of organisms was tested for their suitability for bioassay research. As a result, in 1992 a smaller number of suitable species was studied in greater detail. In that year the terrestrial part of the study was also started. An overview of the first two-year field survey under practical agricultural conditions is presented in De Jong & Bergema (1993). In 1993 the bioassays were developed under more controlled conditions (glasshouse, experimental field) and in 1994 the most suitable bioassays were repeated once.

The results were presented at the SETAC-conferences in Lisbon and Houston in 1993 (De Jong & De Wit, 1993; Bergema & Walgreen, 1993; De Jong & De Snoo, 1993) and the results of the bioassays with *Pieris brassicae* and *Chaoborus spec.* were presented at the International Symposium on Crop Protection of the University of Gent (De Jong & Van der Nagel, 1994; Bergema & Rombout, 1994).

1.2 Objective and problem formulation

The aim of this study is:

> **To design, validate and standardize field trials for evaluating the direct and indirect side-effects of pesticides in the aquatic and the terrestrial environment.**

To this end the following research questions have been formulated:

1. **Is it possible to trace the direct and indirect side-effects of pesticides in the field?**
2. **What method is most suitable for tracing these side-effects?**
3. **Is it possible to develop standardized field trials for pre- and post-registration?**

The next section summarizes the methods used to answer these questions.

1.3 Method

Bioassays

The following options are available for studying pesticide side-effects in the field (*cf.* De Jong *et al.*, 1990): i) full-scale field studies, ii) bioassay studies with organisms brought into the field in enclosures, and iii) taking substrate from the field into the laboratory for studying toxic effects. Although the third option is not concerned with a field situation, it can be used as a supplementary tool.

A full-scale field study represents the real field situation best. It is characterized by a considerable amount of natural variation, however, and the results are dependent on the organisms that happen to be present. The second option forms an intermediate between field and laboratory trials. Compared to the full-scale situation, bioassays have the advantage that the same organisms can be used in the same quantity at different locations; moreover, organisms can be observed in time series, before and after application of a pesticide. These considerations led to bioassays being chosen for this study.

By following the bioassays in time, the effects of the different treatments can be studied. Supplementary laboratory and glasshouse experiments were conducted as a control on the field results and to demonstrate causality between the field results and the presence of pesticides.

Habitats

Non-target organisms may be exposed to pesticides in several habitats. For the aquatic environment, the subject of study was the emission from the parcels to surface water. In The Netherlands the ditches adjacent to the parcels stand the greatest risk of contamination with pesticides; the aquatic part of the study therefore focused on these ditches. Later, enclosures in untreated ditches adjacent to untreated pastureland were used.

For the latter part of the research, enclosures with controlled rates of pesticide application were used and experiments conducted in microcosm units at Wageningen Agricultural University. The advantage of the use of enclosures in natural ditches is that the differences in a number of environmental conditions (e.g. oxygen, pH, nutrients and current) are much lower in the enclosures than in different natural ditches. The microcosm research provides an opportunity to validate the bioassays under highly controlled conditions.

In the terrestrial environment non-target organisms may be exposed both inside and outside the parcels. The highest exposure rates will occur inside the parcel, giving the highest risk of side-effects. In the Netherlands, however, there is an ongoing discussion about the occurrence of non-target species inside the target area. In view of the diversity of ideas on this subject, we choose to conduct our side-effect field study outside (adjacent to) the agricultural parcels.

Experiments were conducted under practical agricultural conditions as well as on an experimental field. On the experimental plot a knapsack-type sprayer was used. The advantage of this method was that experiments could be conducted at the most suitable moment (in terms of weather conditions, etc.). In actual practice the farmers decided whether and when to use the pesticides, which on several occasions led to experiments without interpretable results.

Test species selection

The selection of test species is extremely important for interpreting results (assessing side-effects). We have selected test organisms representing the different trophic levels of the ecosystem, i.e. primary production, herbivores, carnivores and decomposition. In this way side-effects can be interpreted at the ecosystem level, indicating potential effects on overall environmental quality.

The following criteria were used for the selection of test species:
- the main emission routes and means of exposure should be covered;

and species should:
- be suitable for examining the main modes of action;
- be related to environmental quality;
- represent different taxonomic groups;
- be common in the habitats examined;
- survive in the bioassay environment;
- not be insensitive to pesticides.

Proceeding from these selection criteria, we postulated that test species should be present from at least the main ecosystem functions: primary production, herbivory, carnivory and decomposition.

The specific criteria and reasons for the results of this selection procedure are discussed in the chapters on aquatic and terrestrial bioassays. In the aquatic environment in the first two years of the study the suitability of a number of species was tested: periphyton algae and duckweeds *Lemna minor*, *Lemna gibba* and *Spirodela polyrhiza* (primary producers), water fleas *Daphnia magna* and pond snails *Lymnaea stagnalis* (herbivores), sticklebacks *Gasterosteus aculeatus* and larvae of phantom midges *Chaoborus crystallinus* (carnivores), and larvae of midges *Chironomus riparius* (herbivore/decomposer), isopods *Asellus* spp., amphipods *Gammarus* spp., and decomposition of American river weed *Elodea* spp. and leaf discs of *Brassica oleracea* (decomposition). The results were found to vary, and in the last two years the study focused on bioassays with periphyton algae, *Lemna* spp. and *Spirodela polyrhiza*, *Chaoborus crystallinus*, and *Gammarus* spp.

Next the most promising species were selected; Table 1.1 summarizes the test species for which a field bioassay has been developed.

Table 1.1 Bioassays developed.

	aquatic	terrestrial
primary producers	*Lemna minor, L. gibba* *Spirodela polyrhiza*	*Brassica napus* *Poa annua*
herbivores	-	*Pieris brassicae*
carnivores	*Chaoborus crystallinus*	-
decomposers	*Gammarus* spp.	litterbags

1.4 Report design

The aquatic and the terrestrial parts of the study are presented in Chapter 2 and Chapter 3, respectively. The bioassays, presented in Table 1.1, are presented in two parts: the results of the field studies and a guideline for conducting the bioassays. In Chapter 4 the results are discussed and conclusions drawn. In addition, proposals are presented for further research, required to improve the reliability of the tests.

2 AQUATIC BIOASSAYS

This chapter presents the methods and results of the aquatic studies. First, the *in situ* bioassay studies performed in 1991 and 1992 are presented (Section 2.1). Then the 1993 and 1994 laboratory bioassay studies, which include toxicity tests and bioassays in model ecosystems (microcosms), and in enclosures in a drainage ditch, are presented (Section 2.2).

2.1 *In situ* bioassays (1991 & 1992)

In this section first the methods of the preliminary studies of 1991 and 1992 are described (Section 2.2.1). The results of 1991 are presented as an overview, while the results of 1992 are presented in more detail (Section 2.2.2).

2.1.1 Methods

Field lay-out & locations

In the vicinity of Leiden, drainage ditches bordering on different crops were investigated. For untreated control, ditches at a minimum distance of 100 metres were used. The depth of the ditches varied from 20 to 40 cm. In some cases in 1992 a 100% treated control was also used, achieved by placing jars with test organisms in the crop and comparing the results with those from jars placed near the untreated control ditches. Figure 2.1 shows the field lay-out. For each species tested, bioassay units were placed at each sample point. Depending on the test species, the number of bioassay units per sample point was one or two (1991) or four (1992). The cropping systems investigated included flower bulb (1991), tree nursery (1991 & 1992), potato (1992), maize (1992) and fruit orchard (1992).

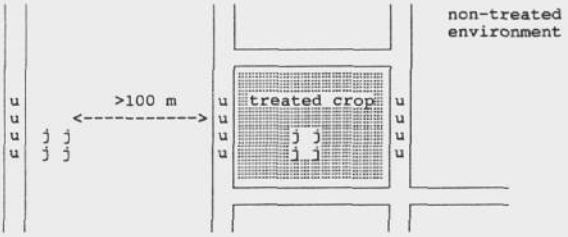


Figure 2.1 General field lay-out for the aquatic *in situ* studies; u=unit with test organisms in the ditch; j=jars for 100% treated control.

Test species

In the first year of study, the suitability of various organisms as test species was investigated. In the second year, a function-based selection was made. The basic point departure

was that the test species should represent each of the main ecological levels. Other criteria were: common occurrence, relative importance, sensitivity to pesticides, and availability of methods for rearing and *in situ* bioassays (see chapter 1). The selected test species are summarized in Table 2.1.

Table 2.1 Test species selected.

Ecological level	1991	1992
Primary producers	periphyton algae	periphyton algae
Herbivores	*Daphnia magna* *Lymnaea stagnalis*	*Daphnia magna*
Carnivores	*Gasterosteus aculeatus*	*Chaoborus crystallinus*
Decomposition	*Asellus* spp. *Gammarus* spp. *Chironomus riparius*	decomposition of *Elodea* spp.

In situ bioassays

For studying periphyton biomass production and species composition in the field, a submerged glass method was used. This method has been well documented and evaluated (Castenholz, 1961; Dumont, 1969; Herder-Brouwer, 1975; Klapwijk, 1980; Tippet, 1970). Microscope glass slides were placed in glass racks wrapped with stainless-steel mesh (1 mm) to avoid grazing by herbivores. The glass racks were fixed in a vertically oriented gauze cylinder (1-mm mesh, 30 cm ϕ), in order to keep the water free of duckweed and filamentous algae. Prior to usage the glass plates were rinsed in a 40% ethanol solution. After four weeks the algae were scraped from the glass surface with a razor blade. Chlorophyll-a contents were analysed according to the Dutch standard method NEN 6520, based on spectrophotometric detection.

The invertebrates and the decomposition of *Elodea* were tested in exposure chambers (Fig. 2.2).

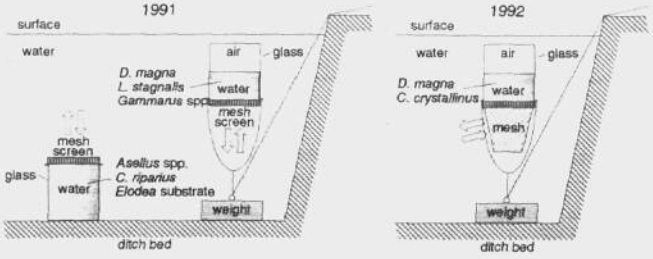


Figure 2.2 Schematic view of exposure chambers for invertebrates and *Elodea* substrate.

The exposure chambers consisted of a 350-ml glass jar with a 0.28-mm mesh (*D. magna* & *C. crystallinus*) or 1-mm mesh (other invertebrates & *Elodea* substrate) stainless steel wire screen in the screwing lid. From 1992 onwards, the method was improved using a 0.28-mm mesh bag in the screwing lid instead of a flat wire screen (fig. 2.2). Exposure chambers for *D. magna* and *C. crystallinus* were placed in the ditch with the wire screen facing downward, enclosing air in the jar. The air provided buoyancy to the construction, and also some oxygen to the test animals when the dissolved oxygen level in the ditch was low. In the laboratory, the water in the improved exposure chamber was completely renewed every fifteen minutes at a continuous flow rate of 0.3 cm/s. Chambers with *Asellus* spp., *C. riparius* and *Elodea* were placed on the ditch bed with the wire screen facing upward, not enclosing air. For *Elodea*, additional holes measuring approximately 2 cm^2 were made in the wire screen, allowing for infestation by invertebrates. Food substrate was added for *L. stagnalis* (fresh *Hydrocharis morsus-ranae*), *Asellus* spp. (dead plant matter) and *C. riparius* (ditch sediment). *G. aculeatus* was tested in galvanized cages (6-mm mesh) measuring 40x25x25 cm. The cages were placed on the ditch bed with the top extending to the water surface, thus allowing the animals to breathe at the surface at low dissolved oxygen levels.

Table 2.2 Properties and responses of the test species recorded *in situ*.

Test organism	Life stage	Origin	Quantity per unit	Responses (time)
periphyton algae	-	field	2 glass plates[e]	chlorophyll-a[g], species-composition[h] (4 wk)
D. magna	neonates (0-2 d)	laboratory[a]	10 (1991) 20 (1992)	survival, size, numbers of eggs and neonates (1 wk)
L. stagnalis	egg (3-4 d)	laboratory[b]	1 clutch of ± 120 eggs	hatching (4 wk)
	juvenile (3 wk)	laboratory[b]	10	survival (4 wk)
G. aculeatus	adult	laboratory[c]	3 ♀, 1 ♂[f]	survival, fresh weight (2 wk)
C. crystallinus	3rd/4th instar	field	10	survival (1 wk)
Asellus spp.	adult	field	10	survival (2 wk)
Gammarus spp.	adult	field	10	survival (2 wk)
C. riparius	2nd instar	laboratory[d]	10	survival (2 wk)
decomposition of *Elodea*	-	field	1.5 gr dried substrate	dry weight loss (4 wk)

[a]culturing method based on NPR 6503, using medium of 2.5 g/l sea salt in demineralised water; initial cultures of *D. magna* and *Chlorella pyrenoidosa* obtained from RIVM, Bilthoven; [b]obtained from Free University, Amsterdam; [c]obtained from Leiden University; [d]obtained from TNO, Delft; [e]microscope slides, 76x26 mm; [f]three cages with one ♂ plus one cage with three ♀ per sample point; [g]according to NEN 6520; [h]identifications were made once in August 1991.

The properties, quantities and responses of the test organisms are summarized in Table 2.2. In order to explain variations in periphyton growth, orthophosphate and nitrate were analysed at regular time intervals. As a measure of food availability for *D. magna*, the chlorophyll-a content of the ditch water was analysed (NEN 6520) at regular time intervals. Dissolved oxygen levels were measured to explain the results for *C. aculeatus*. The oxygen measurements were made twice: once in the afternoon and once at sunrise.

Pesticide exposure estimates and analyses

The maximum pesticide levels in the exposed ditches were estimated from wind speed and direction, the accompanying spray drift percentages (De Snoo & De Wit, *in prep.*) and a standard ditch depth of 25 cm. The spraying rates were supplied by the cooperating farmers; for wind speed and direction during spraying, measurements from De Bilt were used, provided by the Dutch meteorological service KNMI, De Bilt.

In 1991 the cholinesterase-inhibiting activity of ditch water was measured once at the flower bulb location and twice at the tree nursery location. From the latter location water samples were taken four days after spraying with acephate and one day after spraying with methyl-parathion. In 1992, the fungicide captan was analysed once at the fruit location in ditch water and in the treated control bioassays one day after spraying.

Treated control bioassays

As 100% treated controls, bioassays with *D. magna* and *C. crystallinus* were exposed to the field rate applied in the parcels (Fig. 2.1). They consisted of 750-ml glass jars containing 500 ml of water sampled from a ditch unexposed to pesticides with ten test animals. Treated control experiments were carried out in a fruit orchard being sprayed with the fungicide captan, and in a tree nursery field being sprayed with the insecticide methyl-parathion (Condor®). Two to six replicates per sample point were used. Survival was recorded at seven days post-treatment. Decomposition of *Elodea* was investigated inside the orchard sprayed with captan three times, using 1.5 grams of dried matter in 500 ml water. Six replicates were used. Effects were recorded after four weeks.

Laboratory bioassays with *D. magna* and toxicity tests with *C. crystallinus*

In 1992 laboratory bioassays were carried out with *D. magna* with water samples from the field locations. This method is essentially different from the field bioassay, since effects in the laboratory are related to the presence of a compound at the moment of sampling, whereas in a field bioassay effects are related to pesticide levels changing during the experimental interval. Water samples were taken from ditches to the laboratory and ten animals were tested in 250 ml *in duplo*. Survival was recorded after one week.

In 1992 a toxicity test was carried out with *C. crystallinus* and methyl-parathion (Condor®) to compare with the results of the field experiments. Water from a pond at the *Hortus Botanicus* of Leiden University was used in 500-ml samples. This pond has no history of pesticide exposure. Methyl-parathion was added to the water samples in duplo at 0.1, 1, 10, 100, 1000 μg/l, and a duplo control was included. In each water sample twenty animals were tested. Survival and behaviour were recorded daily during one week. At the start and end of the experiment the dissolved oxygen levels were measured; the saturation levels exceeded 90%.

Investigations into mortality of *D. magna* in unexposed *in situ* controls
In the field bioassay the survival in untreated situations was often rather low. In order to improve the performance of the field bioassay, it was attempted to find the cause for the high mortality in untreated controls. Therefore, in 1992 a factorial research in the field was started. The influence of the following factors were investigated: pH, temperature, adaptation of the test animals to the ditch water, number of animals per exposure chamber and size of the exposure chamber. Each of these experiments is described below.

On sunny days the photosynthetic activity in eutrophic drainage ditches may be high. As a consequence, the pH may reach high levels. Therefore, on a sunny day at noon the pH was measured at the sample points of the potato location. The measurements were compared with the tolerance of *D. magna* to high pH values.

The effects of variations of temperature in the field were investigated using closed jars containing ditch water and test animals. Five jars each containing twenty animals were placed in a ditch and five jars in the laboratory. Survival was recorded after one week.

The effect of stepwise adaptation to ditch water was investigated by transferring half of a test population directly into exposure chambers containing ditch water, and the other half after twenty minutes' adaptation time in a 1:1 mixture of laboratory medium and ditch water. Four exposure chambers with twenty animals per treatment were used. Survival was recorded after one week.

The effect of density in the exposure chambers was investigated by comparing the following treatments: eight chambers containing five animals, four chambers containing ten, two chambers containing twenty, and one chamber containing 40 animals. Survival was recorded after one week.

The effect of the size of the exposure chamber was investigated by comparing the survival of twenty animals in commonly used chambers (Fig. 2.2) with the survival of 100 animals in cylindrical chambers made of wire screen (40 cm high, 8 cm ϕ), placed on the ditch bed and extending to the surface. Two ditches were investigated, using one cylindrical and five common chambers per ditch. Survival was recorded after one week.

2.1.2 Results

2.1.2.1 Overview for 1991

Table 2.3 gives an overview of the results of the *in situ* bioassays.

Periphyton algae
The submerged glass method was found to be readily applicable. Chlorophyll-a measurements were analysed with two-way ANOVA (analysis of variance), using pesticide treatments and time as independent variables and excluding interaction effects (insufficient data). No effects of the applied pesticides were found. This was indeed to be expected, since only insecticides (acephate, parathion and pirimiphos) and fungicides (maneb, thiophanate, thiram and triadimefon) were applied.

The species composition of the periphyton showed very little similarity among sample points, even among sample points located in the same ditch. These results are an indication of high spatial heterogeneity within drainage ditches. No correlations with pesticide use were found.

From these results is was concluded that, due to high spatial heterogeneity, species composition is not likely to be useful as a response parameter for evaluating the side-effects of pesticides in the field. In order to evaluate chlorophyll-a as a response parameter, further research is required in herbicide-exposed situations.

Table 2.3 Summary of the results of 1991.

In situ bioassay	Evaluation of method	Effects of pesticides
periphyton algae	potentially suitable	no effects of insecticides (acephate, parathion, pirimiphos) and fungicides (thiophanate, thiram, triadimefon) found; herbicides not investigated
Daphnia magna	low survival in untreated controls; growth and reproduction variable	no effects of insecticides (acephate, parathion, pirimiphos) and fungicides (thiram, triadimefon) found
Lymnaea stagnalis	hatching of eggs highly variable; survival of juveniles potentially suitable parameter	not tested
Gasterosteus aculeatus	survival very low during warm periods due to low oxygen levels	no effects of insecticides (acephate, parathion, pirimiphos) and a fungicide (thiram) found during cool periods
Asellus spp.	survival variable, probably due to oxygen deficiency	not tested
Gammarus spp.	potentially suitable	not tested
Chironomus riparius	survival unknown; many larvae lost in sediment layer	not tested

Daphnia magna

In the bioassay with *D. magna* high mortality was found in unexposed ditches. It is unlikely that low oxygen was a cause, since *D. magna* can survive at very low oxygen levels (Weider & Lampert, 1985).

Survival and reproduction were analysed by means of two-way ANOVA (analysis of variance), with time and pesticide treatments as independent variables and excluding interaction effects (insufficient data). No significant effects of pesticide treatments were found. In addition, the cholinesterase-inhibition analyses showed no elevated activity in the ditches under investigation.

From these data it is not clear whether there was no exposure at all from the pesticides sprayed, or that no effects occurred at the exposure levels, or that effects could not be traced because of a wide variation in survival, growth and reproduction. It was therefore concluded that further research should be carried out to investigate the wide variations in untreated situations and after actual exposure of the test animals to the pesticides.

Gasterosteus aculeatus
During a warm period very high mortality occurred at all sites. Measurements of the dissolved oxygen level at sunrise during this period showed values as low as 0.04 mg/l. On the basis of the oxygen measurements and the negative correlation between survival and temperature it was concluded that mortality was caused primarily by oxygen deficiency. Because of the oxygen deficiency it was not possible to investigate the potential side-effects of pesticides during the warm period.

Survival and fresh weight in the cooler periods, in which mortality rates were low, were analysed in separate one-way ANOVAs (analysis of variance) with pesticide treatment as the independent variable. No effects of the applied insecticides were found.

Since no cholinesterase-inhibiting activity was found in the ditches (see also the results for *D. magna*), it is not clear whether there was no exposure at all from the pesticides sprayed, or that the sticklebacks were not sensitive to the insecticides at these levels, or effects were not seen owing to low survival in the warm period. The problems encountered with sticklebacks led us to conclude that they are not suitable for use *in situ* in bioassays.

Lymnaea stagnalis, *Asellus* spp., *Gammarus* spp. and *Chironomus riparius*
Experiments with snails *Lymnaea stagnalis*, isopods *Asellus*, amphipods *Gammarus* and mosquito larvae *Chironomus riparius* were carried out to obtain an indication of their potential suitability as bioassay test organisms. During these tests no pesticides were applied.

L. stagnalis juvenile snails seem to survive well in the bioassay over a period of four weeks. There was wide variation in egg hatching, however; in two clusters of eggs the hatching rate was over 90%, whereas in the other three clusters no eggs hatched within four weeks. The mechanism underlying this observation remains unclear. From this experiment it was concluded that juvenile *L. stagnalis* are suitable for an *in situ* bioassay.

Survival of *Asellus* was highly variable. In half the exposure chambers no animals survived, in two chambers 50% survived and in three chambers all animals survived. In the chambers with zero survival a layer of sediment was deposited on the wire screen; this was not the case for the chambers in which survival was found. It is therefore highly probable that the mortality occurred mainly as a result of oxygen deficiency. Blockage of the screen wire can be prevented by placing the chamber in the ditch with the screen facing downward. From this experiment it was concluded that, despite the methodic imperfection, there is scope for developing an *in situ* bioassay with *Asellus*.

Gammarus survived well in the exposure chambers. To provide a more natural environment and food for the animals, *Aesculus* leaves (Taylor *et al.*, 1993) can be added to the chambers. It was concluded that *Gammarus* is potentially suitable for an *in situ* bioassay.

With *C. riparius* the separation of the larvae and the sediment appeared to be a problem. Only 30% of the animals could be retrieved. Alternatively, another substrate, e.g. glass pearls, can be used. This is less representative for the field situation, however. From this experiment it was concluded that *C. riparius* is less suitable for use in an *in situ* bioassay.

2.1.2.2 1992 Studies

Primary producers: periphyton

Results

Periphyton production, measured as chlorophyll-a, is presented in Table 2.4. The results were analysed by means of two-way ANOVA, with time and pesticide treatments as the independent variables, excluding interaction effects (insufficient data). For the data from the potato location this was followed by the Student-Newman-Keuls procedure (multiple range test). Homogeneity of variances was checked with Bartlett's test (Sokal & Rohlf, 1981). The tests were performed on log(X*1000+1) transformed data, where X is the original value. The results shown in Table 2.4 are the retransformed values. Prior to the ANOVA tests, correlations between chlorophyll-a and phosphate levels in the ditch water were calculated: no correlations were found.

Table 2.4 Results of the *in situ* bioassay with periphyton; f=fungicide; h=herbicide; means followed by * are significantly different from other treatments at $\alpha=0.05$.

Crop	Treatment	Average chl.-a ($\mu g/cm^2$)	95%-range ($\mu g/cm^2$)	Number of analyses
potato	- untreated	2.37	2.07-2.71	32
	- maneb[a] (f)	2.34	1.85-2.95	12
	- diquat[b] (h)/ maneb[a] (f)	0.84*f	0.54-1.31	4
maize	- untreated	1.75	1.52-2.02	19
	- atrazine/ bentazone[c] (h)	1.14*	0.79-1.66	4
fruit	- untreated	2.47	1.70-3.59	8
	- thiophanate[d] (f)/ pyriphenox[e] (f)	1.88*	1.11-3.18	4

[a]Maneb-80®; [b]Reglone®; [c]Laddok®; [d]Topsin M® wettable powder; [e]Dorado®; [f]mortality of emergent ditch vegetation was also observed.

In situations where herbicides had been applied, a significant reduction in chlorophyll-a production was found: approximately 65% reduction with diquat/maneb and 35% reduction with atrazine/bentazone. In the case of herbicides the glass plates were exposed

after approximately two (diquat) to three weeks (atrazine/bentazone) of colonization. The maneb treatment alone did not affect chlorophyll-a contents. Rough estimates of the maximum pesticide levels in bordering ditches were: 55-385 μg/l for maneb, 35 μg/l for thiophanate, 50 μg/l for pyriphenox, 20 μg/l for diquat and 15 μg/l for atrazine and bentazone.

Discussion

The strongest reductions in chlorophyll-a contents were found when diquat was applied for destruction of potato haulms. No other field studies with this herbicide have been found reported with which to match our observations. Even so, diquat is considered highly toxic to algae (CTB, 1992). Our field observations seem to comply with this view.

In pesticide toxicity research with periphyton communities, there has been considerable focus on atrazine. In enclosures Hamilton *et al.* (1987) and Herman *et al.* (1986) found chlorophyll-a reductions of 21% and 68% at 35 to 47 days after treatment with atrazine at 80 μg/l and 100 μg/l, respectively. Jurgensen & Hoagland (1990), on the contrary, did not find any reduction of cell densities after two pulse dosages of 100 μg/l atrazine in a stream. Toxic effects of atrazine on periphyton communities are also found when other response parameters are used, including carbon uptake (Hamilton *et al.*, 1987; Herman *et al.*, 1986) and species composition (Hamilton *et al.*, 1987; Kosinski, 1984). Chlorophyll-a is easier to measure, however.

For bentazone it is unlikely that this herbicide contributed to the chlorophyll-a reduction, since it is considered to have only low toxicity to algae (CTB, 1990b).

Conclusion

A reduction of periphyton production was found in herbicide-exposed ditches. These results formed the basis for further testing of the bioassay in the laboratory and in enclosures in a drainage ditch (section 2.2).

Herbivores: *Daphnia magna*

Results

Table 2.5 presents the results of the *in situ* bioassays. Survival in the potato crop was analysed by means of two-way ANOVA, with time and pesticide treatments as independent variables and excluding interaction effects (insufficient data). Homogeneity of variance was checked with Bartlett's test (Sokal & Rohlf, 1981). All other results were analysed by means of Kruskal-Wallis analysis of variance (inhomogeneous variances) with pesticide treatment as the independent variable (Siegel & Castellan, 1988). No differences were found between treatments, except for effects on reproduction with a captan/pyriphenox treatment. In most cases the survival in the water samples, taken weekly from exposed and control ditches to the laboratory, was higher than 80%. No differences were found between treated and untreated ditches, indicating that no pesticides were present in the water samples that are toxic to *D. magna*.

Table 2.5 Results of the *in situ* bioassays with *D. magna*; f=fungicide; i=insecticide; h=herbicide; *significantly different from the untreated group at $\alpha=0.05$.

survival

Crop	Treatment	Avg. (%)	95%-range (%)	Number of cages
potato	- untreated	29.1	24.0- 34.3	119
	- maneb[a] (f)	46.5	27.9- 65.2	12
	- dimethoate[b] (i)/maneb[a] (f) /mancozeb/cymoxanyl[c] (f)	49.1	26.1- 72.1	8
maize	- untreated	60.0	51.8- 68.2	58
	- atrazine/bentazone[d] (h)	77.5	42.5-112.5	4
fruit	- untreated	32.1	19.2- 45.0	20
	- captan[e] (f)/pyriphenox[f] (f)	47.1	16.0- 78.2	4

growth (length)

Crop	Treatment	Avg. (%)	95%-range (%)	Number of cages
potato	- untreated	2.7	2.6- 2.8	78
	- maneb[a] (f)	2.7	2.4- 3.0	11
	- dimethoate[b] (i)/maneb[a] (f) /mancozeb/cymoxanyl[c] (f)	3.0	2.6- 3.4	7
maize	- untreated	3.3	3.1- 3.6	50
	- atrazine/bentazone[d] (h)	3.7	3.0- 4.5	4
fruit	- untreated	2.9	2.7- 3.1	16
	- captan[e] (f)/pyriphenox[f] (f)	2.8	2.4- 3.3	3

reproduction (number of eggs + number of neonates per surviving animal)

Crop	Treatment	Avg. (%)	95%-range (%)	Number of cages
potato	- untreated	1.5	1.0- 2.1	85
	- maneb[a] (f)	1.6	-0.3- 4.4	11
	- dimethoate[b] (i)/maneb[a] (f) /mancozeb/cymoxanyl[c] (f)	2.0	-0.2- 3.3	7
maize	- untreated	5.2	3.7- 6.7	50
	- atrazine/bentazone[d] (h)	5.9	0.5-11.3	4
fruit	- untreated	1.1	0.8- 1.4	16
	- captan[e] (f)/pyriphenox[f] (f)	0.4	-0.3- 1.1	3

[a]Maneb-80®; [b]Brabant®; [c]Topper®; [d]Laddok®; [e]Captan® 83WP; [f]Dorado®.

In the field bioassay the survival in untreated situations was rather low, especially at the potato and fruit location. These disappointing results gave rise to a factorial investigation. From the laboratory bioassays it had already become clear that the unknown factor or combination of factors only act *in situ*. The pH values at the potato location on a sunny day at noon ranged from 7.4 to 9.1, which is well within the tolerance limits for *D. magna* (Frear & Boyd, 1967). In both the temperature experiment and the medium-adaptation experiment no differences in survival were found between the treatments. In the density experiment, it was expected that increasing the density may have a negative effect on survival due to competition for food (phytoplankton). Unexpectedly, in exposure chambers containing the lowest density (five animals per chamber) survival was significantly lower than in the chambers with the higher densities ($P<0.01$, χ^2-test; Siegel & Castellan, 1988). In the field bioassay the density was twenty animals per chamber. Thus, in the *in situ* bioassays density had no negative effect on survival. In the experiment comparing survival in large-sized cylindrical exposure chambers with the regularly used chamber, it was expected that in the large chamber survival would be higher owing to the increased freedom of vertical migration. Significant differences were found between the two chamber types within ditches ($P<0.01$, χ^2-test), but overall the survival rates were equal. It can thus be concluded that none of the factors investigated alone had contributed to the low survival in control ditches.

For captan (fruit) and methyl-parathion (tree nursery) treated control experiments were carried out. The results are presented in Table 2.6.

Table 2.6 Results of treated control experiments with *D. magna*; the fungicide captan (Captan® 83WP) was investigated in fruit, and the insecticide methyl-parathion (Condor®) in a tree nursery.

Compound & time of spraying	Treatment	Captan analysis at day 3 (µg/l)	Survival at day 7 (%) avg. ± st.dev.	Number of jars
captan[a] at day 2	treated	1.4	37 ± 29	6
	untreated	none found	58 ± 29	6
parathion[b] at day 1	treated	-	0	4
	untreated	-	93 ± 5	4

Survival in the captan experiment was analysed by means of ANOVA (Sokal & Rohlf, 1981). No significant effect of 1.4 µg captan/l (measured 24 hours post-treatment) was found. Methyl-parathion was highly toxic, which is shown by the 100% mortality in the treated group and low mortality in the untreated group. On the basis of the area and water content of the jars, and the rate at which methyl-parathion was sprayed, a concentration of approximately 200 µg/l was estimated. In the laboratory, increased mortality was found in water samples from the treated jars that were diluted up to ten times. It was expected that toxicity would also be found in samples diluted 100 or more times, since methyl-parathion is extremely toxic to *D. magna* (48-hr EC_{50} 0.14 µg/l; Mayer & Ellersieck, 1986). This could have been due to the fact that in the parcel it was not the pure

compound that was sprayed, but an encapsulated formulation (Condor®), which causes a slow release of the active ingredient. Therefore, the true methyl-parathion levels could have been (much) lower than the estimated levels.

Discussion

In situ bioassays with *D. magna* were also used in a fruit-growing area in the North-East Polder (Anonymous, 1990), and are being used in the glasshouse area of Westland (oral comm. M. Gorter, Water Board of Delfland). Those studies show that *D. magna* can be successfully applied in *in situ* bioassays and that effects can be demonstrated, most likely caused by pesticides. The question arises why in our studies *D. magna* could not be successfully applied. The study in Westland is being carried out in larger waterways than those in our studies; in addition, the Westland study uses animals approximately ten days old, whereas we used animals at most two days old. Both factors may have a positive influence on the survival in untreated control situations. We cannot find an explanation for the difference in success between the study in the North-East Polder and our study, however.

Conclusion

After two years of field study, there was still a lack of control over survival of the test animals in unexposed situations. From these studies it has become clear that it will be very difficult, if not impossible, to develop a field test with *Daphnia magna* for evaluating the side-effects of pesticides. Therefore, this species was no longer used in bioassay studies.

Carnivores: *Chaoborus crystallinus*

Results

A preliminary *in situ* bioassay was carried out at the tree nursery location. During the test period no pesticides were sprayed. The survival after one week was 90% to 100%.

At the tree nursery location a treated control experiment was also carried out with methyl-parathion. The result is presented in Table 2.7.

Table 2.7 Result of treated control experiment with *C. crystallinus* in tree nursery treated with the insecticide methyl-parathion (Condor®); $n=4$ jars per treatment.

Treatment	Survival at day 6 (%) avg. ± st.dev.
Methyl-parathion treated	0
Untreated	83 ± 13

On the basis of the area and water content of the jars, and the rate at which methyl-parathion was sprayed, a concentration of 200 µg/l was estimated. This rate was highly toxic to *Chaoborus*, which is shown by the complete mortality in the treated group and

fairly high survival in the untreated group. In the laboratory, increased mortality was found in water samples from the treated control that were diluted up to 100 times.

Conclusion

The results show that *Chaoborus crystallinus* can be kept well alive in an *in situ* bioassay and that this species is sensitive to methyl-parathion, a commonly used insecticide. It is therefore concluded that *C. crystallinus* is potentially suitable as a test organism. This species was consequently investigated further in the 1993 & 1994 studies (Section 2.2).

Decomposition: *Elodea* as a substrate

Results

This bioassay was only preliminary tested in ditches. In bioassays carried out at the potato and maize location, infestation of the substrate with invertebrates appeared to be a major problem. At the end of the experiment the substrate had to be separated from invertebrates and faeces, a virtually impossible task. For this reason the remaining substrate could not be weighed accurately, resulting in highly variable calculations of dry weight loss.

For captan (fruit) a treated control experiment was carried out over a period of four weeks, during which fungicide was applied three times. In this experiment no invertebrates were present in the substrate and there were consequently no separation problems. The results are presented in Table 2.8.

The results were analysed by means of a one-way ANOVA (Sokal & Rohlf, 1981). There was no significant difference in dry weight loss, although loss in the captan-treated group was slightly lower. However, visual observation indicated a clear difference in decomposition rate between the treated and untreated group. Apparently, dry weight loss is not a sensitive parameter for characterizing effects on decomposition.

Table 2.8 Results of the treated control experiment at 28 days; n=6 jars per treatment.

Treatment	Dry weight loss (%) avg. ± st.dev.
Captan treated (3x)	51.2 ± 10.3
Untreated	57.0 ± 13.2

Conclusion

In the experiment methodological as well as sensitivity problems were encountered. It is therefore concluded that there is little chance of developing a sensitive test for decomposition with *Elodea* as a substrate. This method was not further tested.

2.2 Bioassays in controlled environments (1993 & 1994)

This section describes the methods and results of the bioassay studies performed in enclosures, at an experimental plot and in the laboratory. First a general description of the methods is given (Section 2.2.1), followed by a presentation of the experiments carried out with the primary producers periphyton algae (Section 2.2.2) and duckweed (Section 2.2.3), the carnivorous midge larva *C. crystallinus* (Section 2.2.4), detritivorous amphipods *Gammarus* spp. (Section 2.2.5) and a pilot experiment with litterbags (Section 2.2.6).

2.2.1 General methods

Test species

Based on the result of the *in situ* bioassay studies, a selection of species was made for further testing under more controlled conditions. Table 2.9 presents an overview of the species selected, experiments carried out and compounds used.

Table 2.9 Test species selected, type of experiments performed and compounds used, applied in commercial formulations.

Ecological level	Test species	Type of study	Compound
Primary producers	periphyton	laboratory	diquat
		enclosure	diquat
	duckweed	enclosure	diquat
		experimental plot	diquat
Carnivores	*C. crystallinus*	enclosure	methyl-parathion
		laboratory	methyl-parathion
		laboratory	diflubenzuron
		microcosm	chlorpyriphos
Decomposition	*Gammarus* spp.	enclosure	methyl-parathion
	litterbags	laboratory	captan

Field lay-out

The outdoor experiments were carried out in enclosures and at an experimental plot. The lay-out of the experimental plot is presented in Figure 2.3. One experiment was performed at the experimental plot with duckweed in 2-l plastic containers placed at various distances downwind from a plot that was sprayed with diquat. For the spraying of the plot a knapsack sprayer was used. A description of the spraying method is given in Section 3.1. All other experiments were performed inside enclosures that were treated with the compounds under investigation (Fig. 2.3).

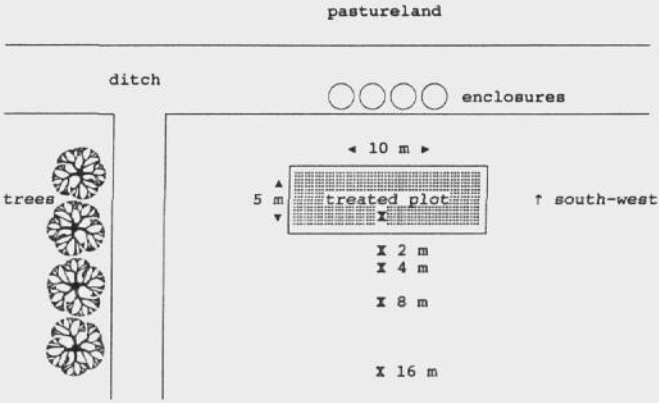


Figure 2.3 Lay-out of experimental plot location; ⧗ = bioassay units with duckweed; the bioassay units inside the enclosures are not shown.

Enclosures

The enclosures were made of white PVC lamellae, which were assembled into cylinders 127 cm wide and 65 cm high. The cylinders were fitted with a polyethylene film on the inside, which was replaced before each experiment. A few days prior to an experiment, the structures were firmly driven into the ditch bed to a depth of more than 10 cm. The mean height of the water column inside was 40 cm, with differences between enclosures of up to 10 cm. The enclosures were covered with wire netting to avoid disturbances by birds and frogs. Spraying of the enclosures with the compounds under investigation was carried out with a plant sprayer when weather conditions were completely calm.

Laboratory toxicity tests

Laboratory toxicity tests were carried out with periphyton and *C. crystallinus* in glass beakers. The results served as a reference to compare with those of the experiments *in situ* and in enclosures. The methods are described in Sections 2.2.2.2 and 2.4.2, respectively.

Microcosms

The bioassay with *Chaoborus* was also tested in a system of twelve microcosm units at Wageningen Agricultural University. This system was highly controlled, and pesticide levels were held constant. The experiments were aimed at effects resulting from long-term exposure to low pesticide levels. The layout of the microcosms is described in Section 2.4.4

2.2.2 Primary producers: periphyton algae

2.2.2.1 Introduction

General aims

The 1993 experiments with periphyton algae had two general aims: i) validation of the correlation found between spraying of the herbicide diquat on a potato field and the reduced periphyton production in a bordering ditch (Section 2.1.2.2), and ii) development of a more readily measurable response parameter. For the first aim, an experiment with diquat was conducted in glass beakers in the laboratory (Section 2.2.2.2) and in enclosures in a polder ditch (Section 2.2.2.3). For the second aim, pilot studies were carried out to investigate various alternatives for chlorophyll-a. The methods and results of the pilot studies are described in this section. Section 2.2.2.4 presents the final evaluation of the periphyton bioassay.

Improvement of parameter response

In a series of pilot studies alternatives for chlorophyll-a as a measure for periphyton biomass production were investigated, including dry weight and optical density of colonized glass plates and ultrasonically fragmented periphyton in aqueous solution. The methods and results of these pilot studies are summarized below.

Dry weight
Dry weight was determined by desiccating colonized glass plates at 60°C, weighing, cleaning and reweighing. For the weighings a mass balance accurate to 0.1 mg was used. The biomass levels could not be determined properly, as they were too near the accuracy level of the mass balance. This method was therefore abandoned.

Optical density of periphyton suspensions
Following De Vries (1986), the optical density of fragmented periphyton in aqueous solution was determined by scraping the periphyton from the glass plates into tap water, sonifying the suspension for 30-60 seconds at 100 Watts (Branson Sonifier B12) and measuring the absorption at 798 nm. The sonification step aimed at breaking down the large chains of cells into small fragments, in order to form a homogeneous suspension. The cell chains were insufficiently fragmented by this method, however, resulting in unstable absorption measurements due to sedimentation of the largest fragments. Therefore, this method was also not further tested.

Optical density of colonized glass plates
Measurement of the optical density of colonized glass plates is non-destructive, allowing for repeated measurements at various time intervals. For this purpose a simple battery-powered optical density meter was developed. This apparatus contained two light-sensitive silicon cells; one cell measured the intensity of the light penetrating a colonized glass plate, and the other cell served as a reference. Prior to each measurement series the apparatus was calibrated, using a clean wet glass plate as a zero-reference. Although this apparatus can be used outdoors in daylight, it was always used in the laboratory under a

60 Watt lamp in order to obtain a constant light quality and intensity. In this set-up the maximum deviation between glass plates was 0.4%. In a pilot study using droplets of *Chlorella pyrenoidosa* suspensions on glass plates, the optical density was well correlated with the cell density. This promising result prompted further testing of the method in the laboratory (Section 2.2.2.2) and outdoors (Section 2.2.2.3).

2.2.2.2 Laboratory toxicity test with diquat

Aims

In February 1993 a toxicity experiment with diquat was performed in the laboratory. The experiment had three aims: i) to validate the *in situ* effect of diquat; ii) to study the feasibility of culturing periphyton in the laboratory for toxicity studies; and iii) to evaluate the usefulness of the optical density of colonized glass plates (Section 2.2.2.1) as a response parameter. In this experiment it was necessary to establish a laboratory culture, since periphyton could not be obtained from the field owing to the winter season.

Methods

Periphyton laboratory culture

Periphyton was cultured in the laboratory in an artificial medium designed for growing *Chlorella* spp. (NPR 6503). A pilot experiment revealed that this medium showed a higher periphyton growth rate than two other growing mediums reported in literature (Alga, growth inhibition test: OECD, 1984; Wood's Hole medium: De Vries, 1986). In order to obtain the initial periphyton population the medium was inoculated with water from a pond at the Botanical garden of Leiden University. The medium was softly aerated. As a substrate for the periphyton the submerged glass method was used (Section 2.1.1). After two weeks the colonized glass plates were used in the toxicity test.

Toxicity test

A toxicity test with periphyton was carried out in three 2.5-l glass beakers filled with 2 l *Chlorella*-growing medium. In two beakers diquat-dibromide (Agrichem®) was added at nominal rates of 20 µg/l and 0.2 µg/l, respectively; in the third beaker no diquat was added, serving as the untreated control. The 20 µg/l concentration was based on a rough estimate made for the maximum diquat concentration in a ditch being exposed due to spraying of the bordering potato field in 1992 (Section 2.1.2.2.). In each beaker twenty pre-colonized glass plates were placed vertically in glass racks. The water was softly aerated to establish a slow water movement and a constant CO_2 level. The temperature varied between 18°C and 21°C and daylight was used. Orthophosphate and nitrate were measured at the start and end of the experiment, showing only minor decreases. Each week five glass plates were randomly selected for measurement of the optical density (Section 2.2.2.1) and chlorophyll-a (NEN 6520).

Statistical analysis

The effects of diquat treatment on optical density and chlorophyll-a at each time interval were analysed in one-way ANOVAs. The correlation between optical density and chlorophyll-a was tested with the Spearman rank-order correlation test (Siegel & Castellan, 1988).

Results

Periphyton responses to diquat

Figure 2.4 shows the periphyton responses to diquat. In this figure the optical densities on day 0 are missing due to initial methodological problems. The ANOVA tests showed no significant ($P>0.05$) differences between treatments at each time interval.

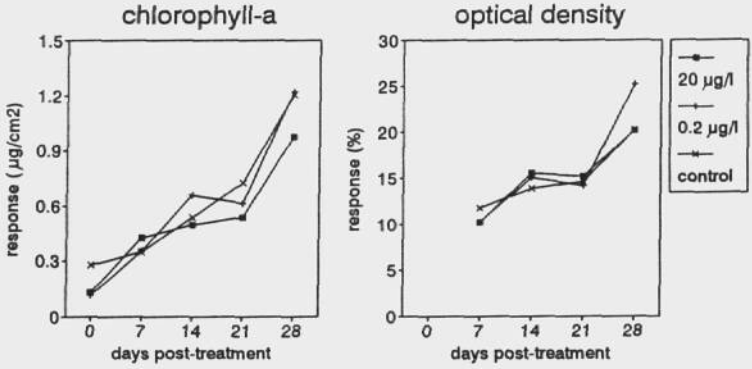


Figure 2.4 Average responses of periphyton to diquat in the laboratory; 5 glass plates per sample mean; no significant differences between treatments were found (ANOVA).

Thus, contrary to the *in situ* bioassay (Section 2.1.2.2), in the laboratory no effects of diquat on the periphyton biomass were found. Various factors may have been contributed to the difference between the laboratory and *in situ* experiments. One possibility is that the herbicide exposure level differed between the two, owing to a bad exposure estimate *in situ*, for example. Another contributing factor may have been a difference in species composition between laboratory and *in situ*, caused by a non-representative species composition in the laboratory periphyton culture, or by differences in available nutrient levels, temperature and light intensity between laboratory and *in situ*.

Correlation between optical density and chlorophyll-a

The correlation between the optical densities of periphyton on glass plates and the chlorophyll-a contents is shown in Figure 2.5. A high correlation (R=0.93) was calculated (Spearman rank-order correlation test). This indicates that optical density may be a good substitute for chlorophyll-a to estimate the periphyton biomass. It should be noted, however, that the optical density measurements of periphyton *in situ* can become distorted

by incorporation of (in)organic particles into the algal biomass. In order to investigate this potential problem, the optical density method was further tested in an enclosure experiment (Section 2.2.2.3).

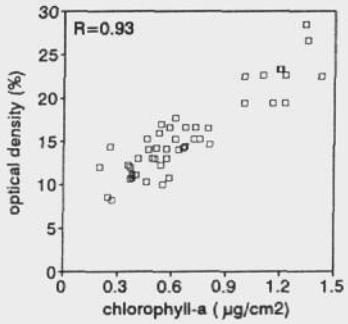


Figure 2.5 Correlation between optical densities and chlorophyll-a contents from paired observations; R=Spearman rank-order correlation coefficient; n=60 glass plates.

Conclusions

The following conclusions can be drawn from the laboratory experiment:

- Culturing of periphyton in the laboratory with artificial medium is well feasible.
- Optical densities correlate well with chlorophyll-a contents, suggesting that optical density may estimate the periphyton biomass as well as chlorophyll-a.
- Diquat applied at 20 µg/l has no effect on the biomass production of laboratory-cultured periphyton. The results with diquat *in situ* could thus not be validated.

2.2.2.3 Bioassay in enclosures

Aims

Despite the fact that the laboratory toxicity experiment with diquat showed no effects, the aims of this experiment were: i) to validate the *in situ* effect of diquat, and ii) to evaluate the usefulness of the optical density in a near-field situation. In this experiment a treated control was also included, in order to determine whether periphyton biomass production shows a measurable response to diquat at all.

Methods

Experimental set-up

The experiment was performed in the period July-August 1993. Three enclosures in a polder ditch were used (Section 2.2.1). The submerged glass method (Section 2.1.1) was

applied, using twenty glass plates per enclosure, 10 cm below the water surface. Glass plates at the outermost positions in the racks were disregarded to exclude edge effects. Possible differences in periphyton between enclosures were eliminated by randomization of the glass plates after 25 days of colonization. Three days later two enclosures were sprayed with diquat-dibromide (Agrichem®): one at 93% (7% lower than intended due to an experimental error; nominal concentration 270 μg/l) and one at 7% (nominal concentration 22 μg/l) of the recommended rate for potato haulm destruction (1000 g a.i./ha). The 7% rate was based on a rough estimate that was made of diquat deposition in a ditch resulting from spraying of a potato field in 1992 (Section 2.1.2.2.). One enclosure remained untreated, serving as a control.

Responses

The chlorophyll-a contents of one half of the glass plates were analysed three days pre-treatment and those of the other half four weeks post-treatment. The glass plates used for the first chlorophyll-a analysis were replaced by new glass plates. The optical density (Section 2.2.2.1) of all the glass plates was measured weekly from three days pre-treatment until four weeks post-treatment in the laboratory. Orthophosphate and nitrate in the enclosures were measured weekly from one day pre-treatment to four weeks post-treatment.

Statistical analysis

Chlorophyll-a contents were analysed per time interval by means of one-way analysis of variance (ANOVA). Homogeneity of variances was checked with Bartlett's test (Sokal & Rohlf, 1981). Optical densities were analysed non-parametrically (inhomogeneous variances) with the Kruskal-Wallis test (Siegel & Castellan, 1988).

Results

Enclosure nutrient levels

The nutrient levels in the enclosures are shown in Table 2.10. Since growth is measured over time, the nutrient levels are presented as averages of the four-week period post-treatment.

Table 2.10 Average enclosure nutrient levels in post-treatment period; n=5 sample dates.

Diquat treatment	Orthophosphate (μg P/l) avg. ± st.dev.	Nitrate (μg N/l) avg. ± st.dev.
93% field rate	696 ± 152	110 ± 94
7% field rate	421 ± 78	41 ± 58
untreated	280 ± 27	76 ± 31

Table 2.10 shows that the average phosphate and nitrate levels differed between the treatments. These differences already existed at the time of treatment, indicating that they were not caused by diquat treatment.

Periphyton responses

The periphyton responses to diquat treatment are shown in Figures 2.6 and 2.7. It should be noticed that at 3 days pre-treatment the chlorophyll-a contents and optical densities are independent, but that at 25 days post-treatment both parameters are paired.

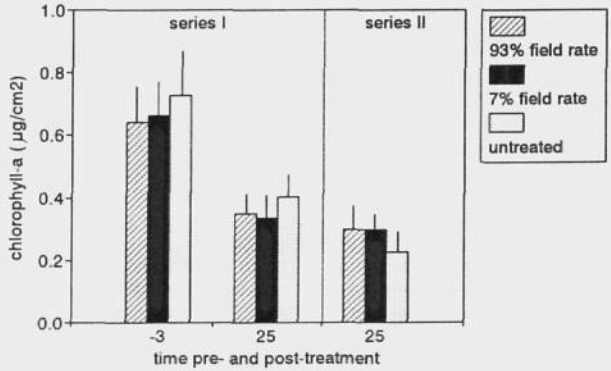


Figure 2.6 Average chlorophyll-a contents of periphyton in enclosures treated with diquat; | =standard deviation; series I: pre-colonized (four weeks) prior to treatment; series II: non-colonized prior to treatment; n=8 glass plates per sample mean; no significant differences between treatments were found (ANOVA).

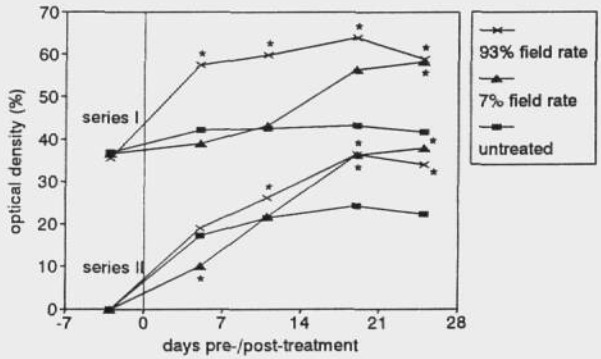


Figure 2.7 Average optical densities of periphyton in enclosures treated with diquat; *=significantly different from untreated control at $\alpha=0.05$ (Kruskal-Wallis); series I & II: see Figure 2.6; n=8 glass plates per series (repeated measurement).

It was expected that at least the highest treatment would cause growth inhibition. This was not the case, however. Moreover, the chlorophyll-a contents showed no differences

between treatments in either series or at any sampling time. The results from the *in situ* bioassay (Section 2.1.2.2) could thus not be validated in this near-field situation.

In the pre-colonized series from one week post-treatment onwards it was seen that the algal pellets became dissociated from the glass plates. This phenomenon is mirrored by decreasing chlorophyll-a contents with time. This indicates that the total colonization period should not exceed four weeks. In the *in situ* bioassays this condition was met.

The optical density responded differently to diquat than did chlorophyll-a content. In the pre-colonized series the optical density in the 93% treatment was consistently higher than in the untreated group. At 25 days post-treatment this was also the case in the 7% treatment. This treatment was comparable to the highest treatment in the laboratory where, on the contrary, no effects were found (Section 2.2.2.2).

Correlation between optical density and chlorophyll-a

The correlations between the optical densities of periphyton on glass plates and the chlorophyll-a contents are shown in Figure 2.8. In this figure the correlations are calculated per series and time interval. This was done since within the set of data three distinct subsets were recognizable, and when tested as one set no correlation was found (Spearman rank-order correlation test); when tested separately, in two subsets significant correlations were found (Fig. 2.8).

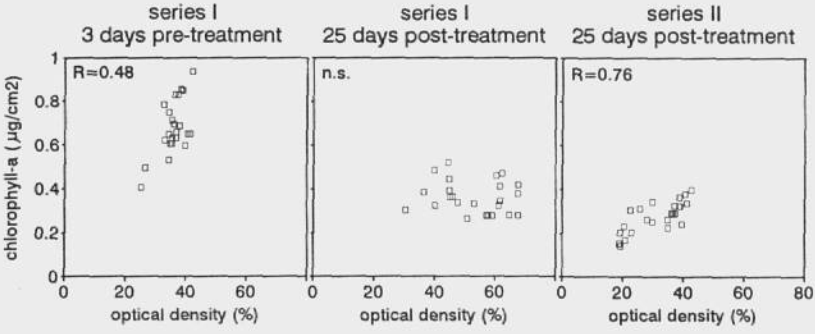


Figure 2.8 Correlations between optical densities and chlorophyll-a contents from paired observations; series I & II: see Figure 2.6; R=Spearman rank-order correlation coefficient; n.s.=no significant correlation ($P>0.05$); n=24 glass plates per correlation test.

These results indicate that no one-to-one relationship exists between the two parameters. One of the factors causing this variability seems to be interference of (in)organic particles with the optical density. This would seem plausible, based on the fact that the oldest periphyton community (the pre-colonized series at four weeks post-treatment) exhibits the highest ratio of variation in optical density and chlorophyll-a content (assuming that the incorporation of particles in periphyton communities increases with age) and no signifi-

cant correlation. It is therefore concluded that, not only because of the risk of dissociation of algal pellets from the glass plates, but also because of the risk of particle incorporation, it is recommended to limit the colonization time to four weeks.

Conclusions

From the enclosure experiment the following conclusions can be drawn:

- Inhibition of periphyton biomass production *in situ* (Section 2.1.2.2) could not be validated in this near-field situation; moreover, 93% of the recommended field rate did not show any effect on periphyton biomass production.
- The optical density of periphyton communities more than four weeks of age may be overestimated due to incorporation of (in)organic particles.
- The colonization of periphyton on glass plates should be investigated over periods limited to four weeks, in order to avoid dissociation of the algae and to minimize the effect of incorporation of (in)organic particles into the communities.

2.2.2.4 Discussion periphyton algae

The laboratory experiment showed no effects of diquat on chlorophyll-a content or optical density at a pulse dosage of 20 μg a.i./l, and in enclosures even at 270 μg a.i./l. These findings are well in line with those of Barreiro-Lozano & Pratt (1994), who found in a laboratory microcosm study that at a pulse dosage rate even thirteen times higher the chlorophyll-a content was not affected. Photosynthetic activity measured via electron transport system activity or oxygen evolution, on the contrary, was inhibited shortly after dosing, but recovered (partly) within a few weeks. Recovery may be explained by the fact that diquat is readily adsorbed by sediments and suspended matter (CTB, 1992). It should, however, be emphasised that such a rate is far above the levels to be expected in the field. It therefore seems justified to conclude that the periphyton community-level responses chosen in our studies are not sensitive to diquat at realistic exposure levels.

The laboratory and enclosure results (no effects at up to 93% of the recommended field rate) are in contrast with the *in situ* bioassay observations (reduced growth in a ditch exposed to diquat spray drift). Possible explanations for this contrast may be traceable to differences in the experimental set-up. Polder ditches are strongly heterogenic, both spatially and temporally. In enclosures, and even more so in the laboratory, heterogeneity is kept low. This of course increases the statistical power of the experiment, but reduces the level of realism. The part or complete isolation of the periphyton community from the ecosystem potentially affects the availability of active ingredient, nutrient levels and the species composition of the periphyton. It is assumed that one or a combination of these factors caused the observed contrast between the bioassays *in situ* and in controlled environments.

With respect to the compound diquat, the promising results with the bioassay *in situ* were not validated in the laboratory and enclosures. Two remarks should be made on these results.

Firstly, the results for diquat cannot be generalised for other herbicides. Although the most pronounced effect *in situ* was seen with diquat, on the basis of its physico-chemical properties this compound may have been an unfortunate choice, since it is known to be readily adsorbed by sediments (CTB, 1992). Atrazine, another compound studied *in situ* that showed an effect, can on the contrary remain in the water column for prolonged periods of time (CTB, 1990a). For this reason the *in situ* results with atrazine might have been easier to validate.

Secondly, the response parameter chlorophyll-a was chosen, not because it was expected to be the most sensitive parameter, but because it is a parameter that can be easily measured and compared between samples. Since it is a community-level parameter, it is expected to be less sensitive than a species-level parameter, such as diversity or abundance of sensitive species. It is well conceivable that, using such parameters, effects of diquat can be observed.

From the periphyton studies no clear indications were obtained that the bioassay in its present form is useful for evaluating the side-effects of pesticides. Subject to the possibility that for other compounds better results might be achieved, at this point the conclusion is drawn that this bioassay is not useful for evaluating the side-effects of herbicides *in situ*.

2.2.3 Primary producers: duckweed

2.2.3.1 Introduction

In polder ditches with high nutrient loads duckweeds are the most common macrophytes. In summer they often form complete blankets covering the ditch from bank to bank. This seriously affects the ditch ecosystem. By shutting off the light and restricting the gas exchange with the air, this results in reduced growth of submerged plants and decreased dissolved oxygen levels (Hillman, 1961). Ultimately, reduced macrofauna diversity may result. Due to these negative effects, duckweeds generally have a poor reputation in the Netherlands.

However, duckweeds can also have positive effects on the ditch ecosystem, by creating a habitat for a variety of invertebrates, including hydras, flatworms, snails, aphids, springtails, bristletails, beetles and spiders; in addition, they form a food source for carp, ducks and muskrats (Hillman, 1961).

Duckweeds may be considered very sensitive to herbicides, as demonstrated by Grossmann *et al.* (1992) in their laboratory studies. They compared the sensitivity of five different plant bioassays to 32 herbicides. The bioassays included growth inhibition tests with algae *Scenedesmus acutus*, duckweed *Lemna paucicostata* (not found in The Netherlands), with callus cells from maize *Zea mays* and oilseed rape *Brassica napus*, and a seed-germinating test with cress *Lepidium sativum*. The *Lemna* test was the most sensitive or equally sensitive in 27 cases (84%).

Compared with other macrophytes, duckweeds in bioassays have many practical advantages. Owing to their rapid growth, responses can be measured quickly. In addition, their small size allows for a large experimental set-up, resulting in high statistical power. Furthermore, duckweeds are well described, culturing methods are available, and their physiology and reproductive biology are well-known (Hillman, 1961). Moreover, a simple method is available for measuring duckweed growth in the field (De Groot *et al.*, 1987).

The practical advantages of duckweeds and their sensitivity to herbicides was the reason for starting investigations into the potential for developing a field bioassay for herbicide side-effects. In 1993 bioassay experiments were carried out with the duckweeds *Lemna minor*, *Lemna gibba*, and *Spirodela polyrhiza*. The experiments were carried out in enclosures in a polder ditch and in containers at an experimental plot using diquat-dibromide (Agrichem®) as a test compound. Diquat is mentioned specifically for controlling duckweed in waterways (Mandersloot, 1993). The duckweeds were obtained from the ditch bordering on the experimental plot. In 1994 the enclosure experiment was repeated in order to assess the reproducibility of the effects. In the enclosures the duckweed was exposed by direct spraying; in the experimental plot the duckweed was exposed via droplet drift from plot spraying. The layout of the experimental plot is given in Section 2.2.1. The enclosure experiments are presented in Section 2.2.3.2 and the field plot experiment is presented in Section 2.2.3.3. The duckweed studies are discussed in Section 2.2.3.4.

2.2.3.2 Enclosure experiments

Aims

The enclosure experiments were performed to test the suitability of duckweed in field bioassays for herbicide side-effects. At the same time, it was attempted to establish whether or not growth, measured via cover rate, is a suitable response parameter.

Methods

Bioassay

The experiments were performed in August 1993 and July 1994. Following De Groot *et al.* (1987), growth was investigated using floating mini-compartments (Fig. 2.9) in enclosures. A unit consisted of 2x2 (1993) or 5x5 (1994) compartments of transparent PVC, each measuring 15x15 cm wide and 6 cm high, and open at the top and bottom. The compartments were fixed on a frame of 18 mm PVC electricity pipe, glued with prefab bends.

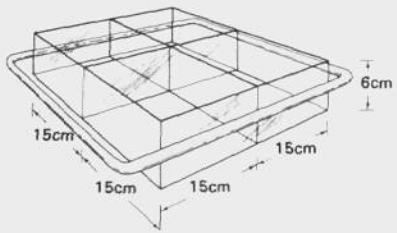


Figure 2.9 Schematic view of a 2x2-compartment floating unit used for duckweed in 1993.

In 1993, a mixture of *L. gibba* and *L. minor* was used. In 1994, *L. gibba*, *L. minor*, and *S. polyrhiza* were tested separately. Each experiment started with a 10% duckweed cover rate. Cover rate was measured to the nearest 5% by pushing the fronds to one side of a compartment with a PVC sheet.

In 1993 three enclosures were used, two of which were sprayed with diquat-dibromide (Agrichem®). One enclosure remained unsprayed, serving as a control. Sprayings were performed at 93% (7% lower than intended due to an experimental error) of the recommended rate for destruction of potato haulms (100 mg a.i./m^2) as a positive control, and at 7% of this rate as a simulation of a droplet drift from spraying of an arable field This rate was based on a rough estimate made for diquat deposition in a ditch during spraying of a potato field in 1992 (Section 2.1.2.2.). In 1994 a 0.7% rate was also included. The layout of the experimental plot is presented in Section 2.2.1. In 1994 a fourth enclosure with a treatment of 0.7% was included.

Duckweed cover was recorded weekly until four weeks post-treatment. In order to avoid competition effects during the experiment, the cover was reduced to 25% (1993) or 10% (1994) if it exceeded 50%. In order to investigate recovery, for compartments with surviving duckweed the growth rate per week interval was calculated, according to the equation:

$$g = \frac{lnC_{t_2} - lnC_{t_1}}{t_2 - t_1}$$

in which g=growth rate (fraction per day), t=day number post-treatment and C=duckweed cover (%).

Statistical analysis

All results were analysed non-parametrically (inhomogeneous variances; Bartlett's test) using the Kruskal-Wallis analysis of variance (Siegel & Castellan, 1988). Duckweed cover was compared between treated groups and the untreated group at seven days post-treatment only, since initial differences remain proportional in the following periods. Growth rates were compared between treated groups and the untreated group per week interval.

Results

The duckweed cover rates are presented in Figure 2.10. In both experiments all duckweed died within one week at the highest treatment rate. At 7 days post-treatment, cover rates were significantly reduced for all species and for all diquat treatments. From the *Lemna* mixture treated at 7%, all *L. gibba* died (visual observation) but *L. minor* remained alive. This suggests a difference in sensitivity to diquat. When *L. gibba* was tested separately, its higher sensitivity compared with *L. minor* was confirmed. *S. polyrhiza* was the most sensitive of all species tested, showing complete mortality during the first week in the 7% treatment and 79% mortality in the 0.7% treatment.

Table 2.11 presents the growth rates of the duckweed that survived the diquat treatment. At the 7% treatment, the growth rate of the *Lemna* mixture was inhibited during the first and second week post-treatment but recovered during the third and fourth week. This response was the net result of *L. gibba* mortality and *L. minor* growth.

In the separate species experiment, at 7% the responses were also clear. *L. minor* showed the same response pattern as the *Lemna* mixture; growth rates were inhibited during the first and second week post-treatment (44% and 22%, respectively) and recovered during the third and fourth week. *L. gibba* showed complete growth inhibition (in fact, partial mortality) during the first week. During the second week growth still seemed to be inhibited (33%) but not significantly, due to a large standard deviation. During the following weeks growth recovered completely. *S. polyrhiza* showed complete mortality within one week.

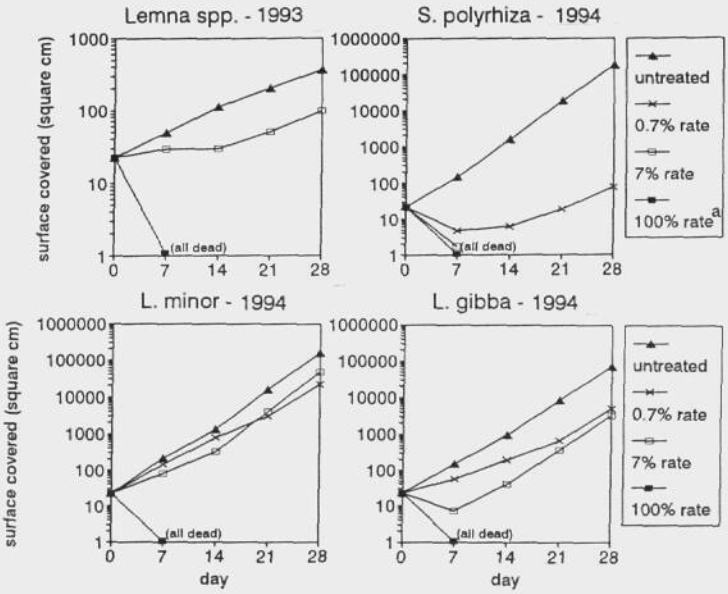


Figure 2.10 Time responses of calculated average cover of a mixture of *L. gibba* and *L. minor* (1993) or individual species (1994) sprayed with diquat (Agrichem®); n=8 compartments per average; at day 7, all treatments differed from the untreated controls (Kruskal-Wallis, $\alpha=0.05$); [a]93% rate in 1993.

Table 2.11 Calculated duckweed growth rates (1/day); n=8, unless otherwise indicated.

Time interval/ treatment	*Lemna* spp. (1993) avg. ± st.dev.	*L. minor* (1994) avg. ± st.dev.	*L. gibba* (1994) avg. ± st.dev.	*S. Polyrh.* (1994) avg. ± st.dev.
wk1 untreated	0.111 ± 0.016	0.319 ± 0.012	0.270 ± 0.021	0.264 ± 0.025
0.7% rate		0.263* ± 0.020	0.121* ± 0.060	-0.210* ± 0.105[c]
7% rate	0.034* ± 0.039	0.180* ± 0.039	-0.169* ± 0.094[b]	all dead
wk2 untreated	0.116 ± 0.023	0.255 ± 0.052	0.253 ± 0.064	0.337 ± 0.013
0.7% rate		0.239 ± 0.023	0.180* ± 0.015	-0.017* ± 0.040[c]
7% rate	0.000* ± 0.000	0.199* ± 0.026	0.170 ± 0.136[b]	
wk3 untreated	0.088 ± 0.015	0.351 ± 0.012	0.313 ± 0.015	0.352 ± 0.014
0.7% rate		0.191* ± 0.014	0.177* ± 0.024	0.157* ± 0.042[c]
7% rate	0.074 ± 0.033	0.359 ± 0.012	0.315 ± 0.042[b]	
wk4 untreated	0.082 ± 0.010	0.321 ± 0.024	0.298 ± 0.024	0.327 ± 0.025
0.7% rate		0.279 ± 0.034	0.280 ± 0.042	0.130* ± 0.081[c]
7% rate	0.084 ± 0.039	0.349 ± 0.008	0.309 ± 0.017[b]	

*significantly different from untreated control at $\alpha=0.05$ (Kruskal-Wallis); [b]n=7; [c]n=6.

At 0.7%, however, the responses of the *Lemna* species were less clear. Growth of *L. minor* was inhibited by 18% during the first week, recovered during the second week, but was unexpectedly inhibited again (46%) during the third week. For *L. gibba* growth was inhibited during the first three weeks (55%, 29%, and 43%, respectively), which is a longer period than at 7%. In addition, at 14 and 21 days yellowish colouring of the fronds was observed. This was not seen at 7% or in the untreated control. These findings suggest that a second, unknown, factor co-contributed to the effects at 0.7%. Therefore, the true impact of 0.7% diquat on the *Lemna* species might have been less than their growth responses suggest.

S. polyrhiza showed complete growth inhibition at 0.7% during the first and second week (in fact, partial mortality), and 55% and 60% inhibition during the third and fourth week, respectively. The unknown factor (see above) may also have played a role for this species. However, during the fourth week its influence was presumably less, since the *Lemna* species was observed to recover completely. These results suggest that a 0.7% diquat treatment severely damages this species for at least one month.

Conclusions

- Cover rate is a suitable response parameter for investigating the effects of herbicides on duckweed growth.
- Of all tested species *S. polyrhiza* is the most sensitive to diquat, showing complete mortality at 7% of the recommended field rate, and severe damage and growth inhibition for at least four weeks at 0.7% of the recommended field rate.
- *L. gibba* exhibits an intermediate sensitivity; it is severely damaged by diquat at 7% of the recommended field rate. After two weeks there is complete recovery of growth. At 0.7% of the recommended field rate there is little mortality; the duration of growth inhibition could not be determined properly, owing to the influence of another growth-inhibiting/limiting factor at this treatment rate.
- Of all tested species *L. minor* is the least sensitive to diquat, showing no mortality and growth recovery after one week at 7% of the recommended field rate.

2.2.3.3 Water containers on an experimental plot

Aims

Additional to the enclosure studies, an experiment was carried out using water containers with duckweeds at various distances from a plot to be sprayed with diquat-dibromide (Agrichem®). In this way the duckweeds were exposed via herbicide drift. An advantage of this method is that the herbicide exposure approximates the real-field situations well. A disadvantage is that the container environment for the duckweed is less realistic than, for instance, the enclosure environment. In this experiment the duckweed species *L. gibba*, *L. minor*, and *S. polyrhiza* were tested separately. This experiment had two aims: i) to establish the sensitivities of the three species to the compound, and ii) to compare cover rate with the more common response parameter dry weight.

Methods

This experiment was performed in August 1993. Polyethylene containers measuring 16x16x8 cm were filled with 1.5 l ditch water. A 10% duckweed cover was added. Duckweed cover was measured to the nearest 5% by pushing the fronds to one side of a compartment with a PVC sheet. Containers with duckweed were placed on an experimental plot at 0 (treated control), 2, 4, 8 and 16 m downwind of the plot to be sprayed (Section 2.2.1). As an untreated control, one group of containers was covered with plastic during spraying. Eight containers per distance and duckweed species were used.

It was attempted to spray the plot at 100 mg/m^2, which is the recommended rate for potato haulm destruction. However, due to an error during preparation of the spraying fluid, the true rate sprayed was 128 mg a.i./m^2 (nominal). For spraying a knapsack sprayer with a four-nozzle spray boom was used. The spraying method is described in Section 3.1. The wind speed during spraying was 2-3 m/s. The droplet deposition was measured using water-sensitive papers placed at each distance. These measurements were rendered invalid, however, because of the onset of drizzle shortly after the spraying event. As an alternative, diquat levels were analysed from the water in the containers (TNO-Zeist). Water samples (2 l, consisting of samples from three containers) were taken 30 minutes after spraying. In order to avoid disturbance by extreme weather conditions (rainfall, wind) or animals, the containers were subsequently placed in a tunnel-shaped tent of transparent polyethylene film with bird nets at both ends.

Duckweed cover rates were measured at one week and two weeks post-spraying. At two weeks, dry weight was also measured by drying the duckweed at 60°C until no further weight loss was observed. The results were analysed per time interval by means of one-way ANOVA, followed by the Student-Newman-Keuls multiple groups comparison procedure (Sokal & Rohlf, 1981).

Results

Diquat analysis and duckweed growth

The results of the diquat analyses are given in Table 2.12. No diquat was detected in the water from containers outside the sprayed plot. These results suggest that the deposition from droplet drift outside the sprayed plot was extremely low due to the low wind speed.

Table 2.12 Diquat levels measured in spray fluid and in water in containers inside and outside the sprayed plot.

sample	diquat (mg/l)
spraying fluid	8300
0 m (treated control)	2.2
2, 4, 8, 16 m	<0.005

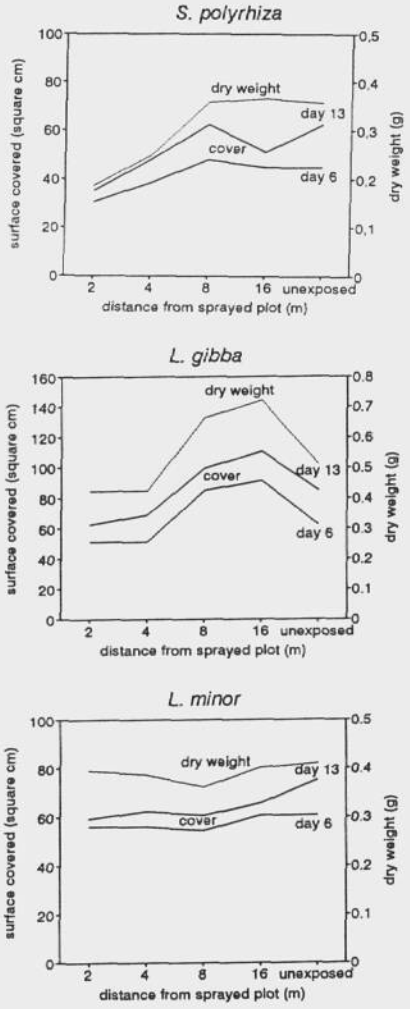


Figure 2.11
Duckweed cover and dry weight at various distances of the sprayed plot; the treated controls (0 m) are not drawn since these showed 100% mortality.

Table 2.13
Statistical test results for duckweed growth at various distances of the sprayed plot.

S. Polyrhiza

stat. test/ distance	surface covered day 6	surface covered day 13	dry w. day 13
ANOVA	**	***	***
SNK			
2 m	a	a	a
4 m	b	b	b
8 m	c	c	c
16 m	bc	b	c
untreated	bc	c	c

L. gibba

stat. test/ distance	surface covered day 6	surface covered day 13	dry w. day 13
ANOVA	***	***	***
SNK			
2 m	a	a	a
4 m	a	a	a
8 m	c	c	c
16 m	c	c	c
untreated	b	b	b

L. minor

stat. test/ distance	surface covered day 6	surface covered day 13	dry w. day 13
ANOVA	ns	*	ns
SNK			
2 m		a	
4 m		a	
8 m		a	
16 m		a	
untreated		b	

ANOVA: ***=$P<0.001$; **=$P<0.01$; *=$P<0.05$; ns=not significant ($P>0.05$); SNK=Student-Newman-Keuls procedure: groups sharing the same letter are not significantly different at $\alpha=0.05$.

Figure 2.11 shows, for each duckweed species, the relationship between the distance from sprayed plot and duckweed cover. The treated controls inside the sprayed plot showed 100% mortality. This treatment was therefore excluded from the statistical analyses. The ANOVA results are presented in Table 2.13.

The results show that the responses of *S. polyrhiza* and *L. gibba* were positively correlated with the distance from the sprayed plot. Reduced growth was found up to 4 metres distance. From the diquat measurements outside the plot (Table 2.12) can be calculated that at all distances the deposition was less than 0.2%. These results suggest that both species are extremely sensitive to diquat.

L. minor showed no differences between the distances, except for the cover at day 13 in the untreated control. This difference does not seem to imply an effect of diquat in the other groups, however, since this effect was not found for dry weight at day 13. These results indicate that *L. minor* is relatively insensitive to diquat.

Cover rate versus dry weight

The cover rates versus dry weights are presented in Figure 2.12. The correlations were calculated and tested with the Spearman rank-order correlation test (Siegel & Castellan, 1988). All correlations were significant (P=0.001).

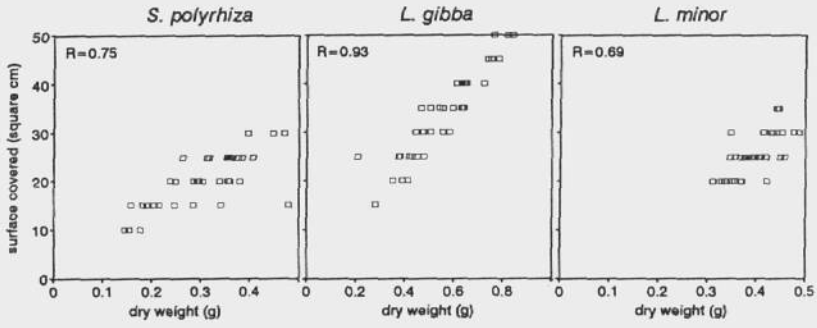


Figure 2.12 Cover rate versus dry weight per duckweed species.

Both parameters are fairly well correlated. Figure 2.12 shows that the correlation is higher when the range of response values is larger; *L. gibba* displays both the highest R-value and the largest response range, whereas *L. minor* displays both the lowest R-value and the smallest response range. This can be explained by the fact that the contribution of measurement error decreases as the range of measurements increases. The high level of significance of the correlations indicate that duckweed cover and dry weight are well comparable. This conclusion is supported by the fact that both response parameters generally yielded the same statistical differences between treatment groups (Table 2.13). Therefore, cover rate is a suitable parameter for investigating the effects of herbicides on duckweed growth.

Conclusions

From the duckweed experiments the following conclusions can be drawn:

- Duckweed cover and dry weight are well comparable, indicating that cover rate is a suitable parameter to estimate duckweed biomass.
- Growth of *S. polyrhiza* and *L. gibba* in plastic containers is inhibited by diquat at deposition rates lower than 0.2% of the recommended field rate.
- The sensitivity of *L. minor* to diquat could not be measured since no effects were found outside the treated plot; however, it is clear that this species is less sensitive than *S. polyrhiza* or *L. gibba*.

2.2.3.4 Discussion duckweed

The duckweed studies show that a simple method can be used to demonstrate effects within one week of exposure to low diquat levels. Funderburk & Lawrence (1963) have demonstrated that in the laboratory *L. minor* is sensitive to diquat levels as low as 0.5 µg/l. Such a concentration would occur after 0.2% deposition in a ditch containing 40 cm of water. In the controlled field situations we did find evidence for sensitivities of this magnitude for *L. gibba* and *S. polyrhiza*, but not for *L. minor*. The greater sensitivity of *S. polyrhiza* compared with *L. minor* was also observed by Blackburn & Weldon (1965) when diquat was added to ponds.

It should be noted that there is a difference in exposure route; we sprayed the diquat directly onto the fronds, whereas Funderburk & Lawrence and Blackburn & Weldon added the compound to the water. Our exposure method might have had more impact on the duckweeds, owing to the direct contact; besides a photosynthesis-inhibiting action, diquat also has a radical action (Worthing & Walker, 1987), resulting in cell-wall destruction.

In order to standardize the plant materials and to reduce the working efforts, consideration may be given to using laboratory cultures. For duckweeds there are already culturing techniques available (e.g. Retzlaff, 1993). It is also possible to culture duckweeds outdoors in ponds or in large enclosures in ditches.

This method also allows for separate investigation of effects via different exposure routes: 1) direct deposition on the fronds, and 2) exposure via the water column. In such an experiment, part of the fronds would be sprayed inside an enclosure, and half this quantity transferred to an unsprayed enclosure. A portion of unsprayed fronds would then be transferred to the sprayed enclosure.

In agricultural practice, deposition rates into ditches of between 1% and 10% droplet drift are likely to occur. Our experiments indicate that at these drift rates some duckweed species are at risk. Our experiments also indicate that these species have a recovery potential. At this point, it is unclear how recovery information should be interpreted, however. This topic is discussed further in Chapter 5.

2.2.4 Carnivores: *Chaoborus* midge larvae

2.2.4.1 Introduction

Chaoboridae are common in natural ponds and ditches in the Netherlands (Parma, 1969a; Steenbergen, 1993). Their life-cycle and behaviour have been studied in detail (Parma, 1969b, 1971), showing that they prey on zooplankton and are important as food for fish. *Chaoboridae* are used in many pesticide toxicity studies (e.g. Apperson *et al.*, 1976, 1978; Hanazato & Yasuno, 1989; Helgen *et al.*, 1988; Leeuwangh *et al.*, 1992; Tidou *et al.*, 1992; Yasuno *et al.*, 1988); the study by Helgen *et al.* (1988) also included a field bioassay.

The promising results from preliminary *in situ* bioassay experiments in 1992 with *Chaoborus crystallinus* (Section 2.1.2.2) prompted further development of this bioassay. This was established in a series of experiments in the laboratory, a semi-field situation, and in real-field situations. Section 2.2.4.2 presents laboratory toxicity tests with the insecticide diflubenzuron (Dimilin®), a blocker of chitin synthesis, and the insecticide methyl-parathion (Condor®), an inhibitor of cholinesterase activity. These compounds were selected because of their different modes of action and their toxicity; both compounds have been reported to be highly effective at low rates for controlling *Chaoborus* populations in lakes (Apperson *et al.*, 1976, 1978; Ali & Lord, 1980). In Section 2.2.4.3 two bioassay experiments in enclosures in a drainage ditch are presented. In these experiments methyl-parathion (Condor®) was sprayed at three rates. In addition to these experiments with exposure from a single dosage, effects due to constant exposure to low pesticide levels were also investigated; bioassay experiments were carried out in replicated laboratory model ecosystems (microcosms) contaminated with either the cholinesterase-inhibiting insecticide chlorpyrifos or the herbicide atrazine. These studies are presented in Section 2.2.4.4. Section 2.2.4.5 presents an experiment investigating the survival of *C. crystallinus* in bioassays in uncontaminated drainage ditches varying in width and the soil type of the beds. The results are discussed in Section 2.2.4.6.

2.2.4.2 Laboratory toxicity tests

Aims

The aims of these experiments were: i) to assess the sensitivities of *C. crystallinus* to parathion-methyl and diflubenzuron, and ii) to select the most suitable response parameter(s) for a field bioassay.

Methods

Toxicity tests

Laboratory toxicity tests were performed with third- and fourth-instar *C. crystallinus* larvae, obtained from spring populations in a pond at the *Hortus Botanicus* of Leiden University. This pond has no history of pesticide exposure. Tests were performed in 750-

ml glass jars containing 500 ml of test medium. The medium was prepared with water from the same pond. Prior to testing, the water was filtered and aerated. For filtering, paper retaining coarse particles was used. A few hours prior to a test, a stock solution of diflubenzuron (Dimilin® Flowable: 480 g a.i./l) or methyl-parathion (Condor®: 240 a.i./l) was made in demineralized water. Dilutions into the test medium were made, resulting in concentrations ranging from 10000 to 0.1 μg/l for diflubenzuron, and from 100 to 0.1 μg/l for methyl-parathion. Concentration ranges were made *in duplo*. The range selection was based on the results of pilot experiments. Test animals were sorted randomly into groups of twenty per jar. The jars were sealed with a screwing lid to avoid evaporation of the test compound. Tests were performed at 20-23°C under natural light conditions. Mortality and immobilization of *C. crystallinus* were recorded at one, two, four and seven days.

Statistical analysis

The results of the toxicity tests in the laboratory and the enclosures were analysed with a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls multiple means comparison procedure. Homogeneity of variances was checked with Bartlett's test. The tests were performed on arcsine square root transformed data (Sokal & Rohlf, 1981).

Calculations for EC_{50} and LC_{50}

EC_{50} and LC_{50} values were calculated by log concentration-logit effect regression, excluding the untreated controls. This model is described by $E=E_{max}/(1+a.e^{(-b.C)})$, in which E is the effect observed, E_{max} is the effect maximum (e.g. 100% mortality), a and b are constants, and C is the test concentration in ln(μg/l). The 95%-ranges for EC_{50} and LC_{50} were derived graphically by linear regression performed on the data plotted against the fitted model. No $L(E)C_{50}$ calculations were made if an effect in the untreated control exceeded 10%, or if the highest test concentration was less than 50%. All statistical tests and regression procedures were performed on a personal computer with STATGRAPHICS® software.

Results

Diflubenzuron

The responses of *C. crystallinus* larvae to diflubenzuron are shown in Figure 2.13; the ANOVA results are presented in Table 2.14. No significant differences between treated groups and the untreated group were found on day one. After two and four days effects were significant from 1 μg/l upwards. After seven days effects in the untreated group had increased, resulting in fewer significant differences with the diflubenzuron-treated groups.

Figure 2.14 presents the effects on pupation and emergence at seven days. The pupae and adults in this experiment had developed from the larvae with which the experiment was started. This figure clearly reflects the moult-inhibiting action of diflubenzuron.

In Table 2.15 the LC_{50} and EC_{50} values for the recorded responses are summarized. The LC_{50} and EC_{50} for immobilization were calculated only at four days exposure, since at the other time intervals the effects were less than 50% in the highest treatment (one and two days), or greater than 10% in the untreated control (seven days).

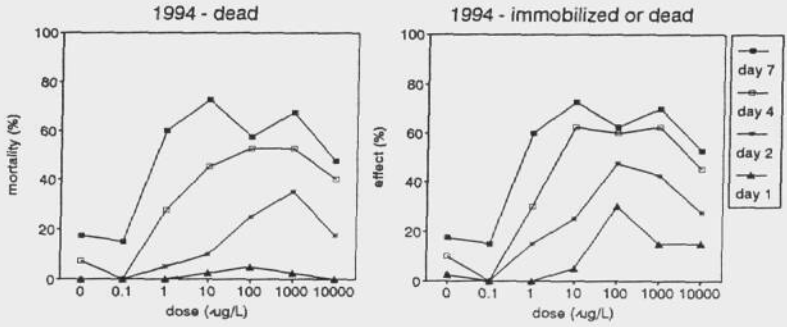


Figure 2.13 Dose-effect relationships for diflubenzuron (Dimilin®) and *C. crystallinus* at various time intervals in the laboratory; n=2 jars per concentration, containing twenty larvae each.

Table 2.14 Statistical test results for the responses presented in Figure 2.13; ANOVA: ***= $P<0.001$; *=$P<0.05$; ns=not significant ($P>0.05$); SNK=Student-Newman-Keuls procedure: treatments sharing the same letter are not significantly different at $\alpha=0.05$.

stat. test/ dose (µg/l)	dead				immobilized or dead			
	1 d	2 d	4 d	7 d	1 d	2 d	4 d	7 d
ANOVA	ns	***	***	*	*	***	***	*
SNK								
0	-	a	b	a	ab	a	b	ab
0.1	-	a	a	a	a	a	a	a
1	-	b	c	ab	a	b	c	ab
10	-	b	cd	b	ab	bc	d	b
100	-	cd	d	ab	b	c	d	ab
1000	-	d	d	b	ab	bc	d	b
10000	-	c	cd	ab	ab	bc	cd	ab

The large 95%-ranges for LC_{50} and EC_{50} at four days show that accuracies were low, however. This was caused by the fact that at concentrations higher than 100 µg/l there was no further increase in the effects with diflubenzuron treatment. This might have been caused by a limited availability of insecticide at the two highest treatment levels. Little precipitate at 1000 µg/l, and more at 10000 µg/l was observed, indicating that the formulation was not fully dissolved in these treatments. Although it was known that the solubility of the technical grade is 100 µg/l in water (Worthing & Walker, 1987), higher concentrations were also tested, since the solubility of diflubenzuron in the Dimilin®

formulation may be different from that of the technical grade. The results of this experiment suggest that this is not the case, however; therefore, toxicity tests with diflubenzuron in Dimilin® should be performed at concentrations not exceeding 100 µg/l.

The EC_{50} values for pupation and emergence were estimated from data that were transformed into percentages of the responses in the untreated control. Compared with the untreated control, it seems that low rates of diflubenzuron stimulate pupation and emergence (Fig. 2.14). This could be avoidance behaviour, but it could also be an artefact. It is concluded that emergence is the most sensitive parameter for investigating the side-effects of diflubenzuron on *C. crystallinus*.

It should be noted that only third- and fourth-instar larvae were used. Early instars may be (much) more sensitive. In this case mortality or immobility could be an even more sensitive parameter. Early instars are difficult to obtain and to handle, however. Therefore, pupa emergence appears to be a more suitable response parameter.

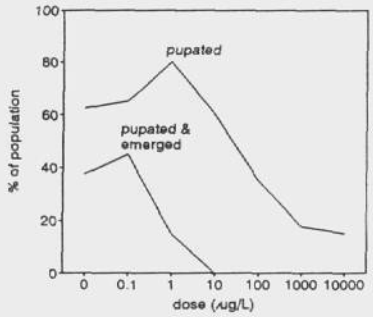


Figure 2.14 Effects of diflubenzuron (Dimilin®) on pupation and emergence of *C. crystallinus* at seven days post-treatment; n=2 jars per concentration, containing twenty larvae each.

Table 2.15 LC_{50} and EC_{50} values calculated from the responses presented in Figures 2.13 and 2.14; 95%-ranges are given in brackets; values are based on nominal rates.

exposure time	L(E)C_{50} (µg/l)			
	mortality	immobilization[a]	pupation	emergence
1 day	>10000[b]	>10000[b]	-	-
2 days	>10000[b]	>10000[b]	-	-
4 days	1880 (123-28800)	138 (4.27-4460)	-	-
7 days	-[c]	-[c]	268 (114-519)	0.95 (0.91-1.00)

[a]immobilized or dead; [b]effect less than 50% at highest test rate; [c]effect in untreated control greater than 10%.

Methyl-parathion

The results of the laboratory toxicity test with methyl-parathion are presented in Figure 2.15, and the statistical test results are shown in Table 2.16. From the responses the LC_{50} and EC_{50} values were calculated; these are presented in Table 2.17.

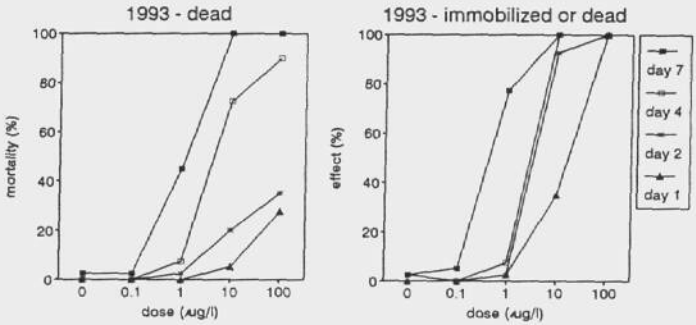


Figure 2.15 Dose-effect relationships for methyl-parathion (Condor®) and *C. crystallinus* at various time intervals in the laboratory; n=2 jars per concentration, containing twenty larvae each.

Table 2.16 Statistical test results for the responses presented in Figure 2.15; ANOVA: ***= $P<0.001$; **=$P<0.01$; SNK=Student-Newman-Keuls procedure: treatments sharing the same letter are not significantly different at $\alpha=0.05$.

stat. test/ dose (µg/l)	dead				immobilized or dead			
	1 d	2 d	4 d	7 d	1 d	2 d	4 d	7 d
ANOVA	**	**	***	***	***	***	***	***
SNK								
0	a	a	a	a	a	a	ab	a
0.1	a	a	a	a	a	a	a	a
1	a	a	a	b	a	a	b	b
10	a	b	b	c	b	b	c	b
100	b	b	b	c	c	b	c	b

At one day exposure the larvae were significantly affected at the 10 and 100 µg/l level; larvae did not respond to tactile stimulation or were dead. As time passed, effects became visible at lower levels. At seven days the EC_{50} was 0.51 µg/l. At one and two days mortality was less than 50% in the highest treatment, so the LC_{50} values were not calculated at these time intervals. At four days mortality suddenly increased, and at seven days the LC_{50} was 1.10 µg/l.

In this experiment effects were better correlated with insecticide level than in the diflubenzuron test. LC_{50} and EC_{50} could therefore be estimated accurately, which is shown by the small 95%-ranges.

Table 2.17 LC_{50} and EC_{50} values calculated from the responses presented in Figure 2.15; 95%-ranges are given in brackets; values are based on nominal rates.

exposure time	L(E)C_{50} (µg/l) mortality	immobilization[a]
1 day	>100[b]	12.9 (21.1-13.8)
2 days	>100[b]	3.92 (3.75-4.10)
4 days	5.21 (4.14-6.57)	1.39 (1.38-1.40)
7 days	1.10 (1.05-1.16)	0.51 (0.41-0.59)

[a]*immobilized or dead;* [b]*effect less than 50% at highest test rate.*

In contrast to the diflubenzuron experiment, in this experiment pupation and emergence were negligible. This difference can be explained by the fact that in the diflubenzuron experiment larvae from an early-spring population (overwintered) were used, whereas in the parathion experiment larvae from the next, late-spring population were used. When early-spring (overwintered) larvae are brought into the laboratory at a high temperatures (simulating summer conditions), they pupate and emerge within a short interval of time; larvae from populations later in the season do not show this response when brought transferred to laboratory conditions.

Conclusions

From the laboratory toxicity tests it can be concluded that:

- *C. crystallinus* larvae are very sensitive to both diflubenzuron and methyl-parathion.
- The dose-effect responses of diflubenzuron were less clear than those of methyl-parathion, however, possibly owing to the low solubility of Dimilin® in water.
- Methyl-parathion is the most suitable compound for outdoor studies, since the responses resulting from this compound (immobilization, mortality) are easier to measure in a field bioassay.

2.2.4.3 Bioassays in enclosures

Aims

The aims of these two experiments were: i) to determine the sensitivity of *C. crystallinus* to parathion-methyl under outdoor conditions, and ii) to assess the reproducibility of dose-effect responses.

Methods

In September 1993 and May 1994 bioassays were carried out in four enclosures in a drainage ditch near Leiden. Each enclosure was stocked with six exposure chambers containing ten *C. crystallinus* larvae each. Third- and fourth-instar larvae were used in the exposure chambers, collected from a nearby ditch.

Using a plant sprayer, three enclosures were treated with methyl-parathion (Condor®), at 48, 4.8 and 0.48 mg/m^2, respectively. These rates correspond to 100%, 10%, and 1% of the rate used in various cropping systems for controlling insects and mites (Mandersloot, 1993). One enclosure remained untreated, serving as a control. At various time intervals methyl-parathion residues in water samples were preconcentrated and analysed using HPLC with UV detection at 280 nm (at the Staring Centre, Wageningen). Water samples were taken from 10 cm below the water surface, the depth at which the exposure chambers were placed. Mortality and immobilization of the larvae were recorded at two, four and seven days post-treatment.

In the second experiment (May 1994), at 3.5 hours post-treatment the water from each enclosure was sampled (three subsamples) through a 0.28-mm stainless steel wire screen. This was used in a laboratory bioassay with *C. crystallinus*. In this bioassay the methyl-parathion levels were also analysed. Mortality and immobilization were recorded at the same time intervals as in the enclosures.

The recorded effects were statistically analysed by means of ANOVA, and LC_{50} and EC_{50} values were calculated; the methods used are described in Section 2.2.4.2.

Results

Parathion analysis

The residual levels of methyl-parathion in the two enclosure experiments and in the water samples from the enclosures used in the parallel laboratory bioassay are given in Figure 2.16. In both experiments no methyl-parathion was measured in the untreated enclosure. The measured residual levels were approximately twenty times lower than the levels calculated from the dose and the water content of the enclosures. The residual levels were highly variable, and did not decrease with time in all cases, which may be expected from sorption and degradation processes. These findings may, at least partly, be explained by the fact that in the Condor® formulation the active ingredient methyl-parathion is encapsulated, causing slow release.

Bioassay responses

The responses of *C. crystallinus* to methyl-parathion in the enclosures and in the parallel laboratory bioassay are shown in Figures 2.17 and 2.18, respectively; the ANOVA results are summarized in Table 2.18. The LC_{50} and EC_{50} values for the recorded responses are shown in Table 2.19. Because of the high variability of the pesticide residue levels, the LC_{50} and EC_{50} values were based on the calculated (nominal) methyl-parathion levels.

Effects in the second experiment were much more pronounced than in the first experiment. In the former, at two days post-treatment mortality and immobilization rates were already higher than in the first experiment at seven days post-treatment (Fig. 2.17). Moreover, the relationship between treatment and effect was much clearer in the second experiment than in the first. This is illustrated by the smaller 95%-ranges for LC_{50} and EC_{50} in the second experiment (Table 2.19). At seven days, in the second experiment the LC_{50} and EC_{50} were at least 50 times lower than in the first. The weaker effects in the first experiment were probably due largely to a lower level of free methyl-parathion (Fig. 2.16).

The results of the laboratory bioassay with water samples from the enclosures at 3.5 hours post-treatment (Fig. 2.18) show high mortality rates in the untreated control, indicating that in the laboratory conditions for *C. crystallinus* were not optimal. Due caution should therefore be applied in interpreting these results. Nevertheless, effects in the laboratory were clearly less pronounced than in the enclosures. Also in this case, the lower effects resulting from the 1% and 10% laboratory treatments compared with the same treatments in the enclosures seem to be due to lower levels of free methyl-parathion. These results give some indication that laboratory bioassays using water samples from the field may not always be representative of the effects occurring in the field.

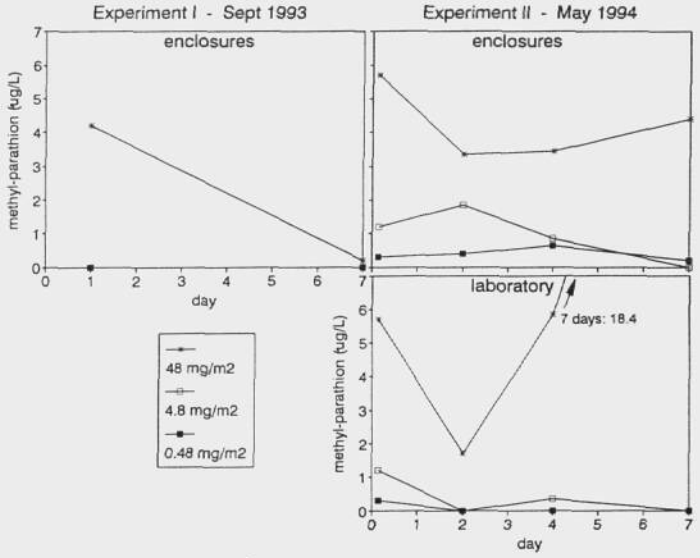


Figure 2.16 Methyl-parathion (Condor®) levels in the enclosures and in water samples from the enclosures used in a laboratory bioassay.

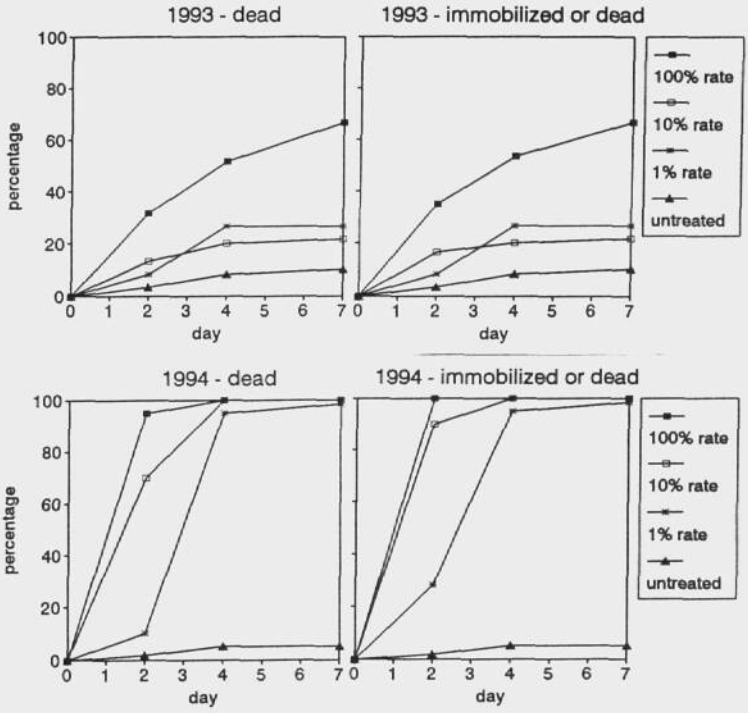


Figure 2.17 Responses of *C. crystallinus* in two bioassay experiments in enclosures treated with methyl-parathion (Condor®) at three rates; n=6 exposure chambers per enclosure, containing ten larvae each.

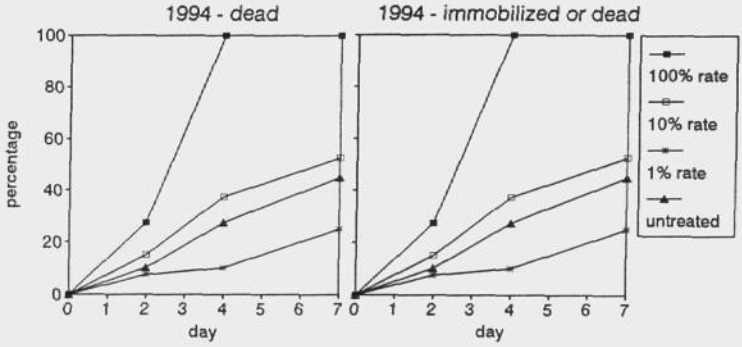


Figure 2.18 Responses of *C. crystallinus* in the laboratory using water samples from the enclosures at 3.5 hours post-treatment during the second experiment; n=2 water samples per enclosure, containing twenty larvae each.

Table 2.18 Statistical test results for the responses shown in Figures 2.17 and 2.18; ANOVA: ***=$P<0.001$; **=$P<0.01$; *=$P<0.05$; ns=not significant ($P>0.05$); SNK=Student-Newman-Keuls procedure: treatments sharing the same letter are not significantly different at $\alpha=0.05$.

statistical test/ treatment	dead			immobilized or dead		
	2 d	4 d	7 d	2 d	4 d	7 d
Exp.I - encl.						
ANOVA	ns	ns	**	ns	ns	**
SNK untreated	-	-	a	-	-	a
1% rate	-	-	a	-	-	a
10% rate	-	-	a	-	-	a
100% rate	-	-	b	-	-	b
Exp.II - encl.						
ANOVA	***	***	***	***	***	***
SNK untreated	a	a	a	a	a	a
1% rate	a	b	b	b	b	b
10% rate	b	b	b	c	b	b
100% rate	c	b	b	d	b	b
Exp.II - lab.						
ANOVA	**	**	*	**	**	*
SNK untreated	a	a	a	a	a	a
1% rate	a	a	a	a	a	a
10% rate	a	a	a	a	a	a
100% rate	b	b	b	b	b	b

Table 2.19 LC_{50} and EC_{50} values calculated from the responses presented in Figures 2.17 and 2.18; the 95%-ranges are given in brackets; values are based on nominal rates.

experiment/ exposure time	$L(E)C_{50}$ (µg/l)	
	mortality	immobilization[a]
Exp. I - encl.		
2 days	>137[b]	>137[b]
4 days	-[c]	-[c]
7 days	49.6 (12.7-193)	49.6 (12.7-193)
Exp. II - encl.		
2 days	6.22 (4.49-8.32)	2.20 (1.58-2.86)
4 days	<1.07[d]	<1.07[d]
7 days	<1.07[d]	<1.07[d]
Exp. II - lab.		
2 days	20.2 (17.0-24.3)	17.9 (15.8-20.4)
4 days	-[e]	-[e]
7 days	-[e]	-[e]

[a]immobilized or dead; [b]effect less than 50% at highest test rate; [c]model fit not significant (ANOVA); [d]effect at lowest test rate greater than 50%; [e]effect in untreated control greater than 10%.

Conclusions

From the enclosure experiments the following conclusions can be drawn:

- The two bioassay experiments in enclosures showed large differences in the responses of *C. crystallinus*, illustrated by the 7-day LC_{50} values (nominal rate), which differed by a factor of at least 50 between the two experiments; some indication was found that this difference may, at least partly, be explained by a difference in the availability of the active ingredient methyl-parathion.
- The effects in the second enclosure experiment are in agreement with the findings in the laboratory toxicity experiment (Section 2.2.4.2), but not with those in the bioassay with water samples from the enclosures; in the latter case it is likely that this was again due to a difference in the availability of the active ingredient.
- Immobilization preceding death was rarely seen in the bioassays in enclosures; therefore, this parameter did not provide much information in addition to mortality, which leads to the conclusion that mortality is the most suitable response parameter in a field bioassay for the side-effects of insecticides via this mode of action.

2.2.4.4 Bioassays in laboratory model ecosystems

Aim

Experiments were carried out in twelve laboratory model ecosystems in order to investigate whether or not the bioassay is suitable for assessing the effects of long-term exposure to low levels of pesticides.

Methods

Microcosms

These experiments formed part of a research project at Wageningen Agricultural University aimed at assessing the direct and indirect effects of long-term exposure to low levels of pesticides, coordinated by T.C.M. Brock of the Staring Centre. The microcosms were made of glass and measured 110x110x70 cm. Each cosm was filled with a 2-cm layer of sediment and 50 cm of overlying water (approx. 600 l), both from the Wezelse Plas at Wijchen. Several mobile macro-invertebrate species from various trophic levels characteristic of Dutch drainage ditches were introduced. For three months the cosms were interconnected by tubes, and the water was circulated by means of a pump. Each cosm was softly aerated. A light-dark regime of 14:10 was maintained, using high-pressure mercury lamps. The room temperature was 19 ± 2°C.

In the microcosms two pesticides were investigated: chlorpyrifos, an organophosphorus insecticide that inhibits cholinesterase activity, and atrazine, a triazine herbicide. When the moment the pesticides were introduced the tubes connecting the microcosms were removed. Chlorpyrifos (Dursban® 4E) was dosed in four enclosures at 0.1 µg/l (0.06-0.13 µg/l). In four other enclosures atrazine was dosed as a pure compound at 5 µg/l (4.2-6.0 µg/l). Four enclosures remained untreated, serving as controls.

Bioassays

In the microcosms three bioassay experiments were carried out. In two experiments a slightly modified form of the field bioassay method was applied: exposure chambers of 750 ml instead of 350 ml in combination with a flat 1-mm wire screen were used, fixed to rods bridging the cosms instead of stone weights (see also Section 2.1.1).

After the first experiment suspicions arose that predatory flatworms that were frequently seen in the exposure chambers were causing mortality of test animals. Therefore, in one experiment 2.5-l glass cylinders (ø 9 cm, length 40 cm) with open tops and bottoms of porous glass membrane were used as exposure chambers. Flatworms were not able to enter these cylinders. The cylinders were fixed to the rods bridging the cosms in such a way that 4 cm protruded above the water surface (effective volume: 2.2 l).

In the exposure chambers third and fourth-instar *Chaoborus* larvae were used. Two experiments were carried out using *C. crystallinus*, collected from a natural ditch near Leiden. One experiment was planned *ad hoc*; this was carried out using *C. obscuripes*, since larvae could be easily obtained from an outdoor experimental ditch of Sinderhoeve at Renkum.

In each microcosm two standard-type (750-ml) exposure chambers were used containing ten larvae each; in the case of the cylinder type, in each of six cosms (two cosms per treatment) one exposure chamber containing twenty larvae was used. The larvae were fed weekly on *Daphnia* spp., starting at day 0. Mortality was recorded weekly.

Statistical analysis

The results were statistically analysed per time interval by means of one-way ANOVA. Homogeneity of variances was checked with Bartlett's test. The tests were performed on arcsine square root transformed data (Sokal & Rohlf, 1981).

Results

The mortality of *Chaoborus* in the three bioassays are shown in Figure 2.19 and the statistical test results in Table 2.20. The results show that within 7 days exposure no significant effects of treatment were found, but that after 14 days or longer exposure times the chlorpyrifos treated groups showed significantly higher mortality. As may be expected from its mode of action (photosynthesis inhibitor), atrazine did not cause effects on survival.

However, the results also show that mortality in the untreated groups was quite high, ranging from 46% to 69%. In the first experiment with *C. crystallinus* using the field-type exposure chamber, predatory flatworms were frequently observed inside the exposure chamber. It was thought that this might have contributed to the high control mortality. Therefore, in the second experiment cylinder-type exposure chambers were used, which kept the flatworms out. In this experiment high control mortality was also found, showing that the flatworms were not the main factor causing mortality in the untreated controls. The best survival in the untreated controls was found for *C. obscuripes*. Apparently, the model ecosystem conditions were not optimal for *C. crystallinus*; to a lesser extent this also holds for *C. obscuripes*. Because of these findings, due caution should be applied in interpreting the effects of chlorpyrifos.

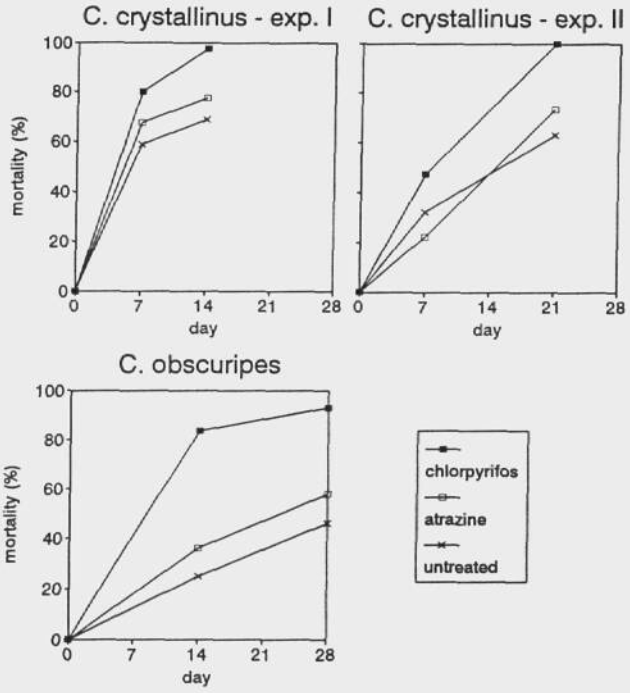


Figure 2.19 Average mortalities of *C. crystallinus* and *C. obscuripes* in bioassays in laboratory model ecosystems (microcosms) with continuous levels of 0.1 µg/l chlorpyrifos or 5 µg/l atrazine, and in untreated systems; the first experiment with *C. crystallinus* and the experiment with *C. obscuripes* were carried out in field-type exposure chambers using eight per treatment (n=8); the second experiment was carried out in glass cylinders using two per treatment (n=2).

Table 2.20 Statistical test results for the mortalities in Figure 2.19; ANOVA: ***=$P<0.001$; *=$P<0.05$; ns=not significant ($P>0.05$); SNK=Student-Newman-Keuls procedure: treatments sharing the same letter are not significantly different at $\alpha=0.05$.

stat. test/ treatment	*C. crystall.* Exp.I		*C. crystall.* Exp.II		*C. obscuripes*	
	7 d	14 d	7 d	21 d	14 d	28 d
ANOVA	ns	***	ns	*	***	***
SNK untreated	-	a	-	a	a	a
atrazine	-	a	-	a	a	a
chlorpyrifos	-	b	-	b	b	b

Conclusion

From the bioassays performed in the laboratory model ecosystems it can be concluded that:

- At the continuous level of 0.1 μg/l chlorpyrifos causes increased mortality of *C. crystallinus* and *C. obscuripes* within two weeks, indicating that the bioassay is also potentially suitable for investigating the effects of continuous exposure.

2.2.4.5 Bioassay in drainage ditches

Aim

The aim of this experiment was to investigate the suitability of the bioassay for use in real-field situations.

Method

In September 1993 the bioassay was tested in six drainage ditches with clay, sandy and peaty beds. On each soil type a narrow (<2 m) and a wide (>4 m) ditch was selected. The purpose of this experiment was to investigate the practical use of the bioassay in various types of water, as determined by soil type and width. Each ditch was equipped with two exposure chambers containing ten *C. crystallinus* larvae each. The larvae were collected from a ditch in the vicinity of Leiden. Survival was recorded at seven days.

Results

Table 2.21 shows that survival in the six uncontaminated ditches was generally high. In one ditch, however, the exposure chambers were lost because a farmer cleaned the ditch mechanically. Thus, no conclusion can be drawn for this ditch. However, in general the results justify the conclusion that water type, characterized by width and soil type of the beds, has no influence on survival. Therefore, the field bioassay can be applied widely.

Table 2.21 Numbers of larvae out of ten living after seven days in exposure chambers in ditches unexposed to pesticides.

	soil type of the ditch bed		
ditch width	peat	clay	sand
<2 m	10; 8	8; 6	-
>4 m	9; 7	10; 8	9; 8

- Exposure chambers lost owing to mechanical ditch cleaning by farmer.

Conclusion

- Overall, *C. crystallinus* larvae can survive well in bioassays in drainage ditches, indicating that the field bioassay is widely applicable in agricultural areas.

2.2.4.6 Discussion *Chaoborus* midge larvae

The responses of *C. crystallinus* in the laboratory toxicity tests are in good agreement with the effects on related species reported in the literature. Diflubenzuron applied in lakes in California (US) at rates ranging from 2.5 to 10 μg/l strongly reduced emergence of *C. astictopus* (Apperson *et al.*, 1978). These observations are in good agreement with our findings that emergence is reduced by 50% at 0.95 μg/l. Methyl-parathion applied to a lake at a rate of 3.3 μg/l effectively reduced *C. astictopus* populations (Apperson *et al.*, 1976). Our 7-day LC_{50} was calculated close to that application rate. The results of our second enclosure experiment with methyl-parathion also agree with these findings, but the results from our first enclosure experiment do not. Differences in effects between the two enclosure experiments seem to be caused mainly by differences in levels of free methyl-parathion.

It appears that even in the controlled environment of enclosures, the fate of methyl-parathion applied as Condor® cannot be readily predicted from application rate and ditch depth. A risk assessment procedure based on the PEC would not be appropriate in the case of Condor®. In this case field bioassays provide more accurate information as a basis for risk assessment, since the effects are based on the actual pesticide levels integrated over time.

Effects that were seen in the second enclosure experiment may also occur in real-field situations. According to the Dutch Commission for the Authorization of Pesticides, droplet drift depositions into ditches from spraying of adjacent fields range between 1 and 10%, depending on the crop (MJP-G, 1991). In our second enclosure experiment at the 1% deposition rate (0.48 mg/m^2) we observed 95% mortality. Although in our enclosure experiments there was no water flow to disperse the compound, similar situations may also occur in practice when the water in a ditch is stagnant. In such 'worst case' situations *Chaoborus* may be at risk.

In the model ecosystems continuously dosed with chlorpyrifos, the bioassay with *C. obscuripes* showed increased mortality at an exposure level that was well below the compound's acute toxicity level (96-hr EC_{50}=0.7 μg/l; Brock *et al.*, 1993). Thus, acute toxicity values may not be valid in situations of continuous exposure. Risk assessment procedures based on acute toxicity tests may therefore not predict risks accurately when pesticide levels are increased continuously; in these situations toxicity tests and field bioassays aiming at long-term effects would be more appropriate.

2.2.5 Decomposers: *Gammarus* amphipods

2.2.5.1 Introduction

Gammarus amphipods are well-studied. Amphipods are very common in the Netherlands (e.g. in North Holland: Steenbergen, 1993). Three species are common in ditches: *G. duebeni*, *G. pulex* and *G. tigrinis*. In the literature, the main focus is on *G. pulex*. These studies show that in freshwater ecosystems they may play an important role as detritivores (Welton, 1979) and as food for fish (Maitland, 1966). They can be easily cultured in the laboratory (McCahon & Pascoe, 1988). Besides mortality, many sublethal responses to toxicants have been studied, including swimming ability (Anderson & Shubat, 1984; Brock *et al.*, 1992), feeding activity (Taylor *et al.*, 1993), growth (Maund *et al.*, 1992), disruption of precopulatory behaviour (Poulton & Pascoe, 1990) and population responses (Maund *et al.*, 1992). Various individual responses have already been successfully measured in a field bioassay (McCahon *et al.*, 1991). Amphipods are relatively very sensitive to a variety of pesticides, such as chlorpyriphos (Brock *et al.*, 1992), flucythrinate (Anderson & Shubat, 1984), lindane (McCahon & Pascoe, 1988), benthiocarb (Sanders & Hunn, 1982) and thiram (Bluzat *et al.*, 1982). These properties make amphipods very suitable for use in a field bioassay for the side-effects of pesticides.

Preliminary bioassay experiments were performed in agricultural ditches in 1991. The results of these pilot studies were promising (Section 2.2.2.1). This bioassay was consequently tested further under more controlled conditions. In September 1993 and May 1994 bioassay experiments were carried out with *Gammarus* spp. in enclosures sprayed with the insecticide parathion-methyl (Condor®) at three rates (Section 2.2.5.2). This compound is an inhibitor of cholinesterase activity. On a small scale the bioassay was also tested in agricultural ditches with different beds and of varying width (Section 2.2.5.3). The bioassay is discussed in Section 2.2.5.4.

2.2.5.2 Bioassays in enclosures

Aims

The aims of the enclosure studies were: i) to determine the sensitivity of *Gammarus* to parathion-methyl under outdoor conditions, ii) to determine whether or not swimming ability is a suitable response parameter, and iii) to investigate the reproducibility of results.

Methods

In September 1993 and May 1994 bioassays were carried out in four enclosures in a drainage ditch near Leiden. Each enclosure was stocked with six exposure chambers each containing ten juvenile *Gammarus* spp. (body length: 3-5 mm in Sept. 1993, 2-4 mm in May 1994) collected from a nearby ditch with a peaty bed. In this type of ditch *G. pulex*

and *G. tigrinus* are the most abundant species. In the bioassays *Gammarus* was fed *ad libitum* on decaying chestnut leafs (*Aesculus hippocastanum*).

Using a plant sprayer, three enclosures were treated with methyl-parathion (Condor®) at 48, 4.8, and 0.48 mg/m^2, respectively. These rates correspond to 100%, 10% and 1% of the rate used in various cropping systems for controlling insects and mites (Mandersloot, 1993). One enclosure remained untreated, serving as a control. At various time intervals methyl-parathion residues in water samples were preconcentrated and analysed using HPLC with UV detection at 280 nm (at the Staring Centre, Wageningen). Water samples were taken from 10 cm below the water surface, the depth at which the exposure chambers were placed. The results of the residue analyses are presented in Section 2.2.4.3. Mortality and immobilization were recorded at two, four and seven days post-treatment; in the first experiment these observations were also made at one day post-treatment.

Statistical analysis

The responses of *Gammarus* were analysed with a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls multiple means comparison procedure. Homogeneity of variances was checked with Bartlett's test. The tests were performed on arcsine square root transformed data (Sokal & Rohlf, 1981).

Results

Figure 2.20 shows the responses of *Gammarus* in the bioassay. The ANOVA results for the bioassay responses are given in Table 2.22.

Table 2.22 Statistical test results for *Gammarus* spp. in the two bioassays in enclosures; $P<0.001$ in all ANOVA tests; SNK=Student-Newman-Keuls procedure: groups sharing the same letter are not significantly different at $\alpha=0.05$.

Treatment	dead				immobilized or dead			
	1 d	2 d	4 d	7 d	1 d	2 d	4 d	7 d
1993								
untreated	a	a	a	a	a	a	a	a
1%	a	a	a	a	a	a	a	a
10%	a	b	b	b	a	b	b	b
100%	b	c	c	c	b	c	b	c
1994								
untreated	-	a	a	a	-	a	a	a
1%	-	a	b	b	-	a	b	a
10%	-	a	c	c	-	b	c	b
100%	-	b	d	d	-	b	c	b

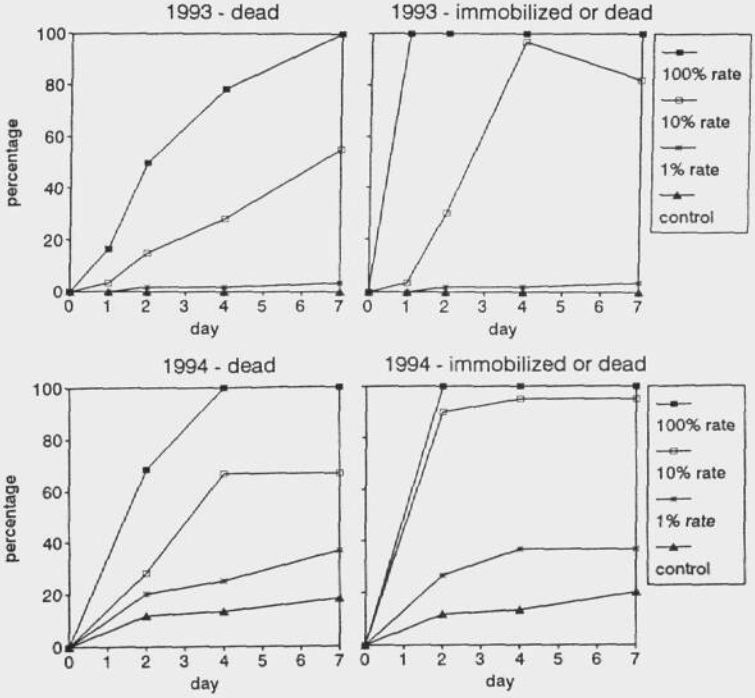


Figure 2.20 Responses of *Gammarus* spp. in two bioassay experiments in enclosures treated with methyl-parathion; 6x10 animals per treatment.

Figure 2.20 shows clear dose-related responses and significant differences between treatment levels (Table 2.22). In the 100%-treated group all test animals were already affected at one day post-treatment in 1993, and at two days post-treatment in 1994. In the untreated control group no effects were found in 1993, but in 1994 mortality amounted to 20% at seven days post-treatment. This mortality can be explained by the fact that the test animals in this experiment were smaller (younger) than in the 1993 experiment. Young animals are generally more sensitive, not only to the compound under investigation but also to other stress factors.

From the bioassay responses LC_{50} and EC_{50} were calculated. The relations of these values with time are given in Figure 2.21. The values are given as nominal initial concentrations, since actual residue levels were highly variable.

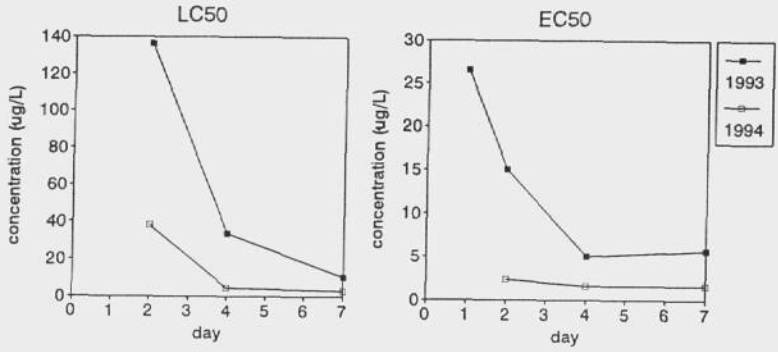


Figure 2.21 LC_{50} and EC_{50} of *Gammarus* spp. as a function of time, based on initial nominal concentrations in the two enclosure experiments.

Figure 2.21 shows that in the 1994 experiment the LC_{50} and EC_{50}, expressed as nominal concentrations, were lower than in the 1993 experiment. It is likely that the differences between the two experiments were caused by the differences in residual parathion levels.

Conclusions

From the two enclosure experiments the following conclusions can be drawn:

- *Gammarus* amphipods are very sensitive to methyl-parathion. Bioassay responses were clearly related to treatment rate; the strongest effects were found in the second experiment.
- Immobilization is a suitable bioassay response parameter that is more sensitive than mortality, especially at short time intervals.
- The dose-effect responses are fairly well reproducible; the differences in effects found in the two experiments can be ascribed to the differences in residual parathion levels.

2.2.5.3 Bioassay in drainage ditches

Aim

The aim of this experiment was to test the suitability of the bioassay for use in various types of agricultural ditch occurring, characterized by ditch width and soil type of the bed.

Methods

The methods used in the *Gammarus* bioassay were the same as those used in the *Chaoborus* bioassay; for a full method description, see Section 2.2.4.2.

Results

Table 2.23 shows the survival in the ditches investigated. *Gammarus* spp. survived well in the ditches with sandy or clay beds, with the exception of the narrow sandy ditch; while the bioassay was being performed, this ditch was mechanically cleaned by a farmer, resulting in the loss of the exposure chambers.

Negative or inconclusive results were found for the ditches with peaty beds. In the wide peaty ditch *Gammarus* had escaped from one of the exposure chambers. In the narrow peaty ditch all test animals died. Preliminary experiments in 1991, however, showed that *Gammarus* spp. can survive well in bioassays performed in ditches with peaty beds. Mortality in this case must have been caused by oxygen deficiency, since this ditch was completely overgrown with duckweed.

Table 2.23 Numbers of amphipods out of ten living after seven days in exposure chambers in ditches unexposed to pesticides.

	soil type of the ditch bed		
ditch width	peat	clay	sand
<2 m	0; 0	10; 10	-
>4 m	9; 0[a]	10; 8	10; 7

[a]two animals were found dead, eight animals had escaped; - exposure chambers lost due to a farmer mechanically cleaning the ditch.

Because of the limited number of ditches investigated, the results summarized in Table 2.23 should be interpreted with great care. In order to test the robustness of the bioassay data, this experiment should be repeated in a larger number of ditches.

Conclusion

From the experiment in various ditches the following conclusion can be drawn:

- The results suggest that *Gammarus* spp. can survive well in ditches with sandy and clay beds in a bioassay; survival in ditches with peaty beds is also expected to be satisfactory if the ditches are not completely overgrown with duckweed.

2.2.5.4 Discussion *Gammarus* amphipods

The results with *Gammarus* spp. are positive. The test animals are easy to handle. Not only mortality but also swimming ability can be easily observed *in situ*. The advantage of sublethal responses is that they are much more sensitive than mortality. *Gammarus* spp. is very sensitive to methyl-parathion and shows clear dose-related responses. Overall, the bioassay seems to be well applicable in agricultural ditches of various types.

In order to increase the sensitivity of the bioassay, it may be worthwhile in future studies to pay more attention to sublethal effects. A simple method to measure the feeding activity *in situ* is available (McCahon *et al.*, 1991); pre-weighed leaf discs of horse chestnut (*Aesculus hippocastanum*), previously 'conditioned' in organically enriched water, are fed during a fixed time interval and re-weighed. Another response tested *in situ* is the disruption of precopulatory behaviour (McCahon *et al.*, 1991); before copulation amphipods form breeding pairs, which become disrupted on exposure to toxicants. Both responses are very sensitive and can be measured at short time intervals. In long-term experiments growth may be a convenient parameter. This can be measured from the ratio between body length and number of antennal segments (Maund *et al.*, 1992).

Although responses at the individual level are easy to measure, it is difficult to interpret the results at the population level or higher levels of ecological organization. For this purpose population responses are more convenient. Maund *et al.* (1992) have used length distribution and population density as responses. From these data the intrinsic growth rate can be calculated, a method developed for *Daphnia magna* (Van Leeuwen *et al.*, 1985). Intrinsic growth rate can be measured *in situ* by means of cohorts representing the age classes of a natural population.

In order to increase the reproducibility of results, it is important to use standardized test animals. McCahon & Pascoe (1988) have developed a simple technique for culturing amphipods. Preliminary observations by ourselves confirm the relative ease with which amphipods can be cultured. When we kept breeding pairs in 2-l water containers with horse chestnut leaves at 20°C, neonates were observed within one week.

The promising experimental results and the positive experiences described in the literature prompt the conclusion that this bioassay is suitable for developing further into a standard field test.

2.2.6 Decomposition: Laboratory pilot study with litterbags

Aim

In a pilot study in the laboratory, litterbags containing dried leaf discs were investigated in the aquatic environment using the fungicide captan. The aim of this experiment was to study the potential suitability of this method, which has been developed for the terrestrial environment, for investigating decomposition in the aquatic environment.

Methods

As stated above, the litterbag method has been developed for the terrestrial environment. For the materials used and the preparation method the reader is therefore referred to Section 3.4.2. The experiment was carried out in 2.5-l glass beakers containing 2 l water from the ditch bordering the experimental plot (Section 2.2.1). In one beaker captan (Captosan® 83-WP) was added at 830 μg/l. This concentration is equal to that resulting in a ditch with 30 cm of water layer if the ditch treated at the recommended field rate (2490 g a.i./ha). Two litterbags containing dried leaf discs of Chinese cabbage (*Brassica oleracea*) were placed in the captan-treated water, and two others in the untreated water.

After seven and eleven days one litterbag per treatment was collected for dry weight measurements of the leaf discs. The results were analysed per time interval by means of one-way analysis of variance (ANOVA). Homogeneity of variances was checked with Bartlett's test (Sokal & Rohlf, 1981).

Results

The results of this experiment are presented in Table 2.24. At seven days a significant difference of almost 9% was found. At eleven days no difference was any longer observed. These results are highly comparable with those of the terrestrial studies, in terms both of the difference and of the decomposition rate.

Table 2.24 Average decomposition of dried leaf discs in litterbags in the laboratory treated with captan at 830 μg/l; n=number of leaf discs; ANOVA: $^{***}=P<0.001$; ^{ns}=not significant.

Treatment	Dry weight loss (%)					
	day 7			day 11		
	avg.	± st.dev.	(n)	avg.	± st.dev.	(n)
captan-treated	77,0***	± 2.8	(19)	92,0^{ns}	± 3.8	(20)
untreated	86,0	± 3.3	(19)	92,9	± 3.5	(20)

Conclusion

From this pilot study it can be concluded that the litterbag method is also potentially suitable for investigating the side-effects of fungicides in the aquatic environment.

3 TERRESTRIAL BIOASSAYS

In this chapter the methods and the results of the terrestrial bioassays are presented. Section 3.1 explains some of the general principles underlying the methods used with all experiments. Sections 3.2 to 3.4 deal with the results of the bioassays with primary producers, herbivores and decomposers, respectively.

3.1 General methods

Cropping systems and pesticides

In 1991 a few preliminary experiments were carried out with *Lactuca sativa* and *Raphanus sativus* as indicators for the effects on primary producers and with litterbags containing cellulose paper as indicators for decomposition. In 1992 a more extended programme was started. An overview of these studies is presented in Table 3.1.

Table 3.1 Cropping systems, pesticides and bioassays investigated in 1991 and 1992.

cropping system	pesticide	bioassay
1991		
potato	DNOC (Luxan oil) diquat (Reglone) buminaphos (Luxan Trakaphon)	Lactuca sativa, *Raphanus sativus*
tree nursery	metham sodium	litterbags
bulb growing	dichloropropene (Shell DD 95)	litterbags
1992		
potato	maneb (Maneb-80)	*Brassica napus* litterbags *Pieris brassicae*
potato	maneb/diquat (Agrichem)	litterbags
maize	atrazine/bentazone (Laddoc)	*Brassica napus* *Pieris brassicae*
orchard	captan (Captan 83% WP)	*Brassica napus* *Pieris brassicae* litterbags
tree nursery	acephate (Orthene)	*Brassica napus* *Pieris brassicae* litterbags
tree nursery	parathion (Condor)	litterbags
bulb growing	glyphosate (Agrichem 2)	litterbags
bulb growing	dichloropropene/metham sodium	litterbags

In 1993 and 1994 tests were conducted in an experimental plot. Table 3.2 presents an overview of the pesticides used and the tests conducted.

Table 3.2 Pesticides and bioassays investigated in the experimental plot in 1993 and 1994.

pesticide	bioassay
bentazone (Agrichem flowable 2) diquat (Agrichem) glyphosate (Agrichem 2)	*Brassica napus* *Poa annua*
captan (Captosan 83 WP) maneb (Brabant)	litterbags
diflubenzuron (Dimilin)	*Pieris brassicae*

Field lay-out

The field trials of 1991 and 1992 were carried out in the vicinity of Leiden. Unexposed controls were situated at least 200 m from the treated field. The field situation generally used is shown in Figure 3.1. Bioassays were placed on either side of a parcel to ensure an exposed range with a certain wind direction. The effects of the pesticide application rate used in practice were investigated.

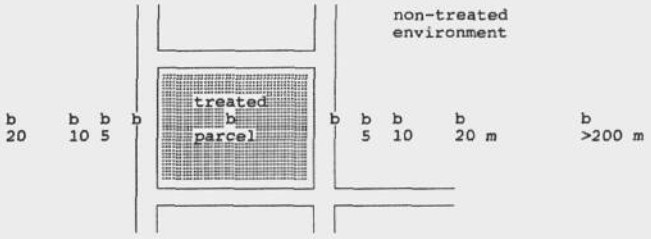


Figure 3.1 Field lay-out for the terrestrial studies in 1992 (b = bioassay unit).

In 1993 and 1994 the field experiments were conducted on an experimental plot (Fig. 3.2). A knapsack sprayer was used for application of the pesticides. The knapsack sprayer was connected to a 1-m spray boom with four nozzles (4110-12). The spray boom was held at 0.5 m from the soil surface. A 10x5 m plot was sprayed in five strips. Spraying pressure was brought to 4 bar at the start of each strip and did not fall below 2 bar during spraying. Deposition of drift droplets was measured at each sample point using three water-sensitive paper strips measuring 2.5x12.5 cm (*cf.* De Snoo & De Wit, 1993).

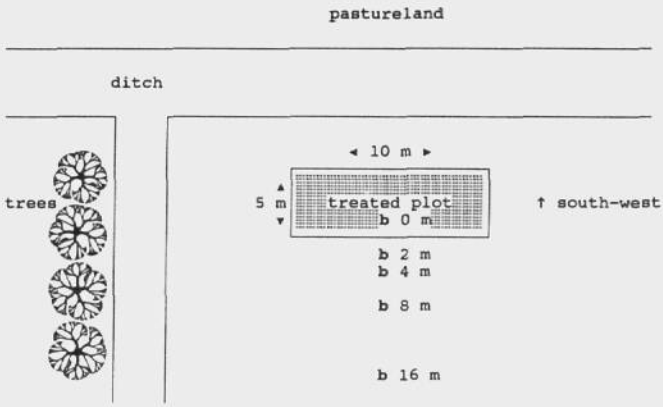


Figure 3.2 Lay-out of the experimental plot; b = bioassay unit.

Statistical analyses

Most results are analyses using an one-way analyses of variance (ANOVA) using the Statgraphics software. This analysis determines whether any differences exists between samples with different treatments. When the ANOVA yielded a significant result a Student-Newman-Keuls a posteriori test was conducted to investigate which groups differ from one another.

The number of replications was determined using the formula (Sokal & Rohlf, 1981):

$$n \geq 2(\sigma/\delta)^2 * \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$$

where

n = number of replications

σ = true standard deviation

δ = smallest true difference required to be detected

v = degrees of freedom

α = significance level

P = desired probability that a difference will be found to be significant

$t_{\alpha[v]}$ and $t_{2(1-P)[v]}$ = values from a two-tailed t-table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively.

This formula was used to determine the number of replications (plants, leaf discs) needed to obtain a 5% difference between sampling points with a 95% reliability. To calculate n, however, it is necessary to know the true standard deviation, so the desired number n was calculated afterwards.

3.2 Primary producers

In this chapter the set-up (Section 3.2.1) and results of the bioassay studies with primary producers are presented. The results are discussed in Section 3.2.3.

3.2.1 Materials and methods

In the terrestrial environment vascular plants are the main primary producers. Other producers such as algae and mosses play a minor role. A bioassay with vascular plants has therefore been developed.

In 1991 one preliminary experiment was conducted in a field situation; in 1992 a number of experiments were conducted under practical agricultural conditions. In 1993 and 1994 the bioassays were improved and tested in an experimental plot. In some cases these outdoor experiments were supported by glasshouse experiments.

3.2.1.1 Preliminary experiment 1991

Test species selection

In 1991 a preliminary experiment was carried out using lettuce *Lactuca sativa* and black radish *Raphanus sativus*. Lettuce *Lactuca sativa* has been used in a number of studies (Hulzebos *et al.*, 1989; Hulzebos, 1990; Breeze & van Rensburg, 1991). The first experiment was carried out in autumn. Since cultivated lettuce plants can be obtained commercially the first experiment was conducted with this species. In the same experiment black radish *Raphanus sativus* was used to study effects on germination. The choice for this species was primarily a practical one. The experiment was conducted in autumn. Black radish is used as an 'organic fertilizer' in this season, so the chance of germination was high.

Tomato *Solanum lycopersicum* has also been used in a number of studies and has been mentioned as a very sensitive species (Straathof, PD, oral. comm.; Breeze & West, 1987a, 1987b; Breeze, 1988a, 1988b). In a number of crops, however, use of tomato plants as test species is not permitted because of the risk of pests; in addition, it does not grow well under autumn conditions.

Field lay-out

The studies by Marrs *et al.* (1991a, 1991b) demonstrate the good feasibility of investigating the side-effects of pesticide drift in the field by means of *in situ* bioassays. In an analogous approach, in 1991 we carried out a preliminary experiment.

Bioassay units were placed in and around a potato field at the time that potato leaves were killed with DNOC (Luxan® DNOC-oil conc., 4 kg a.i./ha), diquat (Reglone®, 1 kg a.i./ha) and buminaphos (Luxan Trakaphon®, 6.4 kg a.i./ha) (Fig. 3.3). The bioassays were placed one day after the treatment with DNOC. Two weeks later both parts of the parcel were treated again, with diquat and buminaphos respectively this time. The grass seed field (sampling point 4) was not treated during the experiment.

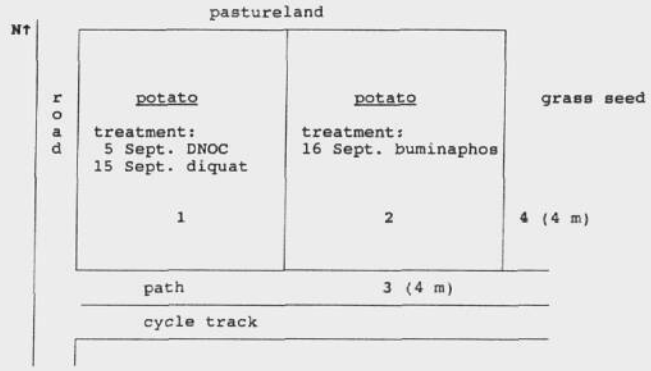


Figure 3.3 Preliminary field experiment with vascular plants; untreated control at >500 m; numbers represent sampling points.

Bioassay unit

Each bioassay unit consisted of a 10-l flower box filled with potting compost. In one half of the box ten lettuce plants were potted and in the other half 30 radish seeds were sown. Two units (1 & 2) were placed in the parcel and two units were placed at four metres' distance (Fig. 3.3).

All units were protected from grazing by birds and mammals using chicken wire. The water supply was arranged via a second box below the unit, connected with jute strips (Fig. 3.4)

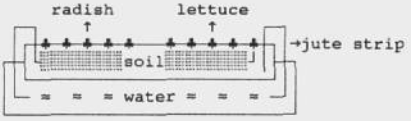


Figure 3.4 Bioassay unit with vascular plants.

At intervals of approximately 5 days visible effects and number of germinated radish seeds were recorded. After one month plants were harvested, root length and leaf surface measured and fresh and dry weight of root and shoot recorded. The results were statistically analysed using one-way analyses of variance (ANOVA).

3.2.1.2 Field experiments 1992

Test species selection

After the first year we looked for more natural species that are not insensitive. The side-effects of drift on natural species have been studied by Marrs *et al.* (1989, 1991). *Lychnis*

flos-cuculi, *Prunella vulgaris*, *Digitalis purpurea*, *Cardamine pratensis* and *Medicago lupulina* appeared to be the most sensitive to herbicide drift. *Cardamine pratensis* and *Lychnis flos-cuculi* are annual plants and their habitat resembles the agricultural environment most closely; these species would therefore seem to be the most suitable as test species. No methods for growing these species under unnatural conditions were available, however.

Oilseed rape *Brassica napus* is also mentioned in the literature, and seems to be even more sensitive (Eagle, 1982). Furthermore, oilseed rape is a crop species, and seeds and growing methods are widely available. This species has become common in rural areas in the Netherlands (Van der Meijden *et al.*, 1983). The combination of commonness and sensitivity makes this species very suitable for use in a field-bioassay. Therefore, oilseed rape *B. napus* was chosen as a test species after the first year.

Bioassay unit
Each bioassay unit contained nine potted plants in separate 9-cm pots with potting compost, placed together in one flower box. In this way it was easier to measure root weight because the roots of the different plants were separated. The bioassay unit (Fig. 3.4) was improved by using a hydro-mat instead of jute, connected to the flower box by means of slots in the bottom of the flower box. The units were covered with 1-cm gauze to prevent grazing.

The plants were grown in the open air and transferred to the field one day before pesticide application. Six days after application plants were taken back into the laboratory, and the fresh and dry weight of shoots and roots measured. The results were tested using a one-way analysis of variance.

Field lay-out
The field lay-out is shown in Figure 3.1. Tests were conducted in maize (June, atrazine/-bentazone, 0.72/0.8 kg a.i./ha), potato (July and August, maneb, 3 kg a.i./ha), fruit (July and August, captan, 1.5 kg a.i./ha) and in a tree nursery (August, acephate, 320 g/ha).

3.2.1.3 Experimental plot 1993 and 1994

In 1993 and 1994 the bioassays were developed further under the more controlled conditions of an experimental plot (Fig. 3.2). In 1993 five field experiments were conducted; in 1994 two experiments were repeated (Table 3.3).

Test species selection
Since monocotyledonous and dicotyledonous species exhibit great differences in sensitivity to herbicides, in the third year a monocotyledonous species was added. *Poa annua* was chosen because it is a common species, easily grown and sensitive to air pollution in general (oral. comm. Van der Eerden, IPO).

In 1993 in two experiments peas *Pisum sativum* were added to study differences in sensitivity between two dicotyledonous species.

Pesticides

The experiments were conducted with three widely used herbicides with different modes of action: diquat (Agrichem diquat®), bentazone (Agrichem bentazone flowable 2®) and glyphosate (Agrichem glyphosate 2®). In one laboratory experiment MCPA (U-46 M-Fluid 500®) was tested. Bentazone and diquat have a photosynthesis-inhibiting action, resulting in yellow and brown leaves, followed by death of the plants (Naber, 1989). Diquat has a secondary destructive effect on the cell walls, which enhances the effect (De Waard *et al.*, 1991). Glyphosate inhibits the formation of amino-acids, resulting in non-selective mortality of all vegetation (Mandersloot, 1993). There are no visible effects such as chlorosis or necrosis (Naber, 1989). MCPA is a systemic hormone-type selective herbicide, readily absorbed by leaves and roots (Worthing & Walker, 1987).

Table 3.3 Overview of the field experiments with plants in 1993 and 1994.

	Brassica napus	*Poa annua*	*Pisum sativum*
glyphosate	25-05-93 06-10-93 12-04-94	01-06-93 06-10-93 12-04-94	01-06-93
diquat	22-07-93	22-07-93	22-07-93
bentazone	21-09-93 03-03-94	21-09-93	

Bioassay units

Experiments were conducted with large numbers of plants in the bioassays. 150 pea and rape seeds were placed in separate compartments in a 150-compartment tray at each sample point. The tray was placed in a transport container and filled with hydro-grains and hydro-mat to ensure a good water supply. In the first experiment the 'old' bioassay unit (Section 3.2.2.2) was used with 60 oilseed rape plants; this experiment was repeated with the 'new' units.

For *P. annua* the improved units (Section 3.2.1.2) shown in Figure 3.4 were used. In these units three parts were sown with 0.075 grams of grass seed. The experiments were conducted with young plants, for oilseed rape when the first real leaves appeared. The use of young plants allowed large quantities to be used; furthermore young plants are generally more sensitive than old plants. Plants were grown indoors. Shortly before spraying they were transferred to the field, and 2-4 hours after spraying they were returned to the laboratory.

In most cases, the initial effects in the 100%-treated unit became visible at one week post-treatment. In these cases, the plants were harvested in three stages, at one, two and three weeks post-treatment. In some cases no effects were visible in the 100%-treated unit after one week; in these cases the first harvest was carried out when the initial effects became visible.

At each time interval 50 oilseed rape and pea plants were harvested. Because the

harvested plants had to be weighed and measured within a short period of time, only 20 oilseed rape and pea plants per unit could be weighed individually; for the other 30 plants the total number surviving and total weight were recorded. For grass, one measurement was performed on 30 grass plants, resulting in 6 values per sample point and time interval.

Statistical analysis

The results were tested using a one-way analysis of variance (ANOVA) to detect any differences in weight between exposure rates. A significant result indicates that results from at least one treatment differ from one other treatment. If the test result was significant, an *a posteriori* test (Student-Newman-Keuls procedure) was conducted to determine which exposure rates differed significantly. To compare the results of the several trials the distance at which a 50% effect was measured was calculated. Because plant weight at the highest dose was not equal to zero, we defined a 50% effect as the effect value midway between the treated and the untreated control values. For calculating plant weight (W), the following model was used (Streibig, 1988):

$$W = (W_{untr}-W_{max})/(1+EXP(-2(a+b\,LOG(dist))))+W_{max}$$

in which W_{max} is the plant weight in the 100%-treated control, W_{untr} is the response of the untreated control, a and b are constants, and dist is the distance from the treated plot. Using a spreadsheet (Quattro-Pro) a best fit of the model to the data was estimated, and the $Edist_{50}$ (Edist = effect distance) value calculated from the resulting model. With this model any stimulating effects at low concentrations are ignored.

At the 50% effect distance the corresponding deposition rate was calculated from the deposition measurements. The deposition was estimated from the three or four sample points nearest to the $Edist_{50}$ according to the model:

$$Deposition = EXP(c-d\,LOG(dist))$$

in which c and d are constants, and dist = distance. Since only a part of the distance-deposition curve was used, this simple model was sufficient for interpolation purposes.

Glasshouse experiments

The field experiments were supported by a number of glasshouse experiments, conducted mainly in the winter. A number of these experiments were preliminary; to examine the suitability of the improved or new units, cultivation possibilities of the species, etc. The dose-response relationships with the herbicides were drawn up as a prediction of the field results. Besides the compounds used in the field experiments (diquat, bentazone and glyphosate) one glasshouse experiment with MCPA was conducted. MCPA has a quite different (hormone-type) mode of action from the other herbicides used earlier. The results of this experiment therefore give an indication of the scope of the bioassay.

The plants for the glasshouse experiments were grown in a climate chamber. The experiments were conducted in an unheated glasshouse. Plants were exposed by means of a plant sprayer. This sprayer appeared to be quite accurate: at each contraction 0.92 ml was sprayed, with deviations of less than 0.05 ml.

3.2.2 Results

3.2.2.1 Preliminary experiment 1991

Bioassay units were placed one day after DNOC treatment; no visible effects were recorded up to two weeks post-treatment. After two weeks the same units were treated with diquat and buminaphos, respectively. In the units treated with diquat, all plants died within 3 days. In the units treated with buminaphos most plants died within one week and all plants died within two weeks. Some radish plants germinated after treatment and survived, however. This results are shown in Figure 3.5.

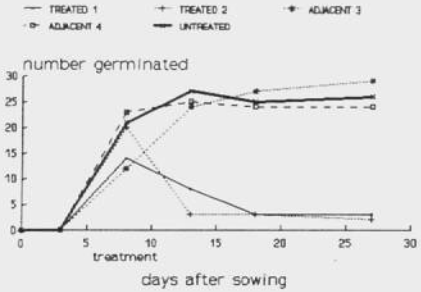


Figure 3.5 Number of germinated radish plants surviving.

There were no visible differences between the units at 4 m from the treated plot and the untreated unit. After harvest, for the untreated unit and the two units at 4 m the dry and fresh weight of root and shoot, the root length and the leaf surface were compared by means of ANOVA. In unit No. 4 the shoots of all radish plants were grazed.

Table 3.4 ANOVA results for lettuce and radish; *** = $P \leq 0.001$, ** = $0.001 < P \leq 0.01$, * = $0.01 < P \leq 0.05$, ns (not significant) = $P > 0.05$; U = untreated; means followed by the same letter in each column do not differ significantly at 5% level according to Student-Newman-Keuls multiple range test.

		shoot			root		
		fresh	dry	survival	fresh	dry	length
lettuce	ANOVA	ns	ns	ns	*	***	ns
	U	-	-	-	a	a	-
	3	-	-	-	b	b	-
	4#	-	-	-	ab	ab	-
black radish	ANOVA	**	*	**	ns	**	ns
	U	b	b	b	-	ab	-
	3	a	a	a	-	b	-
	4#	ab	ab	ab	-	a	-

Due to grazing most shoots in unit no. 4 were destroyed.

The results of the statistical analyses are presented in Table 3.4. For lettuce only the root weight of unit No. 3 was significant higher than the untreated unit. For radish, in contrast, the shoot in unit No. 3 was found to be smaller and weighed less than that in the untreated unit. Root dry weight appeared to be higher in unit No. 3 than in unit No. 4; this could be explained by grazing of the shoot in the latter unit.

Conclusions 1991

There were clear effects on the directly exposed bioassay units; no clear effects were found in the units next to the treated plot. The preliminary experiments showed that this bioassay is feasible and that the test species can be kept alive in the field.

3.2.2.2 Field experiments 1992

The results (Figs. 3.6 to 3.12) show that in one case of herbicide use (atrazine/bentazone) plants near the treated plot suffered side-effects (Fig. 3.6). Within the plot, the directly exposed plants all died. Outside the plot, differences in plant size were seen, and were measured in plant biomass as well. In another parcel an effect was found only within the treated field (Fig. 3.7).

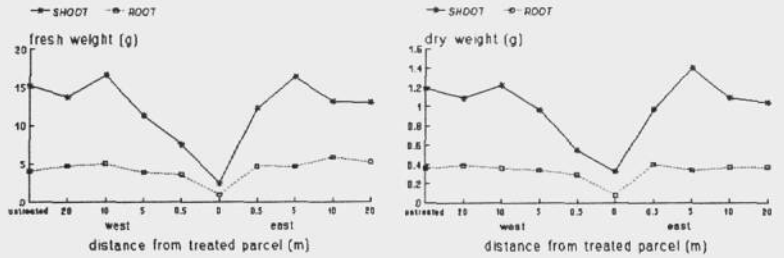


Figure 3.6 Effects of a atrazine/bentazone treatment in maize 03-06-92 on *Brassica napus*; wind speed 3 m/s, west.

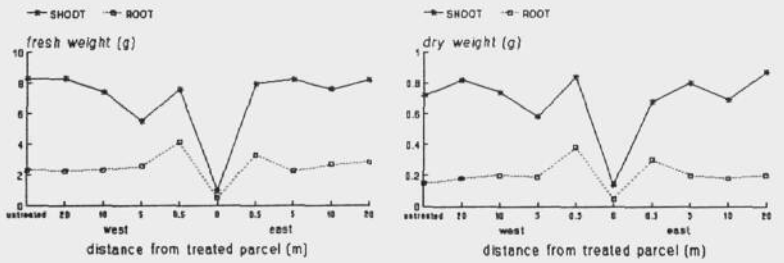


Figure 3.7 Effects of a atrazine/bentazone treatment in maize 03-06-92 on *Brassica napus*; wind speed 3 m/s, west.

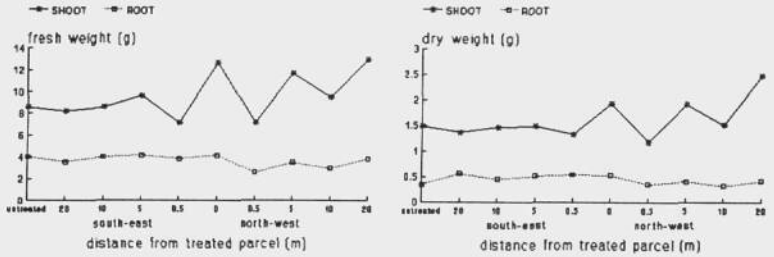


Figure 3.8 Effects of a maneb treatment in potato 06-07-92 on *Brassica napus*; wind speed 8 m/s, north-east.

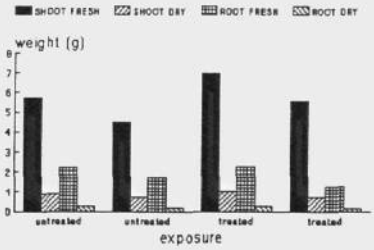


Figure 3.9 Effects of a maneb treatment in potato 11-08-92 on *Brassica napus*; wind speed 5 m/s.

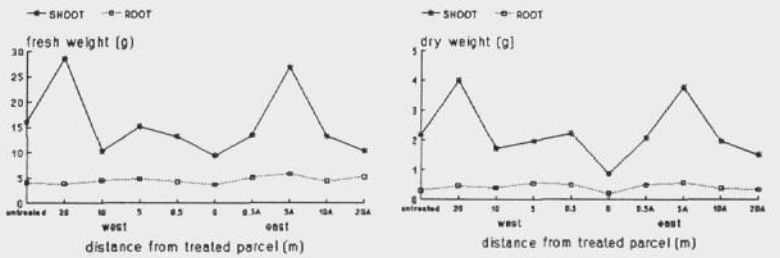


Figure 3.10 Effects of a captan treatment in fruit 09-07-92 on *Brassica napus*; wind speed < 2 m/s; A = units placed shortly *after* treatment.

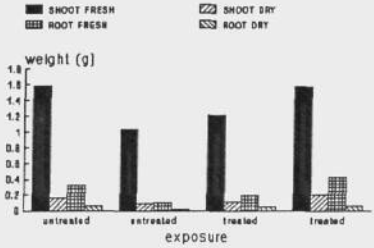


Figure 3.11 Effects of a captan treatment in fruit 27-08-92 on *Brassica napus*; wind speed 5 m/s.

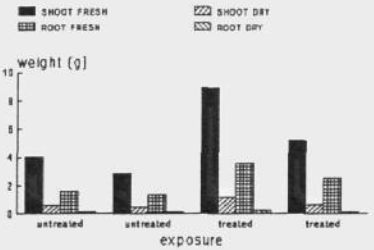


Figure 3.12 Effects of an acephate treatment in a tree nursery 08-08-92 on *Brassica napus*; wind speed 2 m/s.

No effects of fungicides were found (Figs. 3.8 to 3.11). In the case of acephate a positive effect was found (Fig. 3.12). This was due to herbivory in the untreated unit, however.

Shoot fresh and dry weights were highly correlated. Root fresh weight was very variable and root dry weight reflected the same trend as the shoot. It can be concluded that shoot fresh weight alone gives a good indication of effects on plant growth for the pesticides used.

Conclusions 1992

After the field trials of 1992 it was concluded that the bioassay with vascular plants is practical and easy to carry out; test results indicate that the side-effects of herbicides can be traced outside the treated plot.

One of the main problems occurring was that, owing to a combination of the natural variation between the plants and the number of plants used, only differences > 30% in biomass production could be traced at $P < 0.05$. Therefore, in glasshouse experiments and in the year following the experiments were aimed at improvement, and other species were added.

3.2.2.3 Glasshouse experiments

DIQUAT

In a preliminary glasshouse experiment a number of methodic questions were dealt with: i) what are the best cultivation conditions, ii) can oilseed rape plants be grown on sand (because of easier separation of soil particles and roots), iii) can tests be conducted in an unheated glasshouse, and iv) can field conditions be simulated by using a plant sprayer?

In order to answer these questions five plant boxes with 20 plants each were grown on potting compost and two boxes on sand with a nutrient supply. The plants on the sand substrate showed much faster growth. There was major variation among the different potting compost boxes, probably due to their position in the climate chamber. The plants were grown at 20°C with a 14/10-hour light-dark period. The plants appeared to be epinastic. Despite the fact that these conditions were sub-optimal, diquat was applied in a range of concentrations.

Because the climate chamber was continuously used for plant growing, it was not possible to conduct the experiments with herbicides there. An unheated glasshouse was therefore used. The effects two weeks after exposure are shown in Table 3.5. The visual effects show a clear correlation with dose; dry and fresh weight, however, showed no such correlation. It is likely that the cause lies in the large variation between the boxes before treatment.

Table 3.5 Dosages of diquat and visual effects on *Brassica napus*.

dosage (mg a.i./m^2)	substrate	effect
20 (= 200 g/ha)	sand	dead plants, rot of roots large variation in effects
10	potting compost	spots on all leaves
5	potting compost	spots on yellow and young leaves
2.5	potting compost	spots on yellow leaves
0.5	potting compost	purple-coloured older leaves, no difference with untreated
0.1	sand	some yellow spots on older leaves, roots smaller, but intact
0	potting compost	purple-coloured older leaves

It was concluded that it is possible to carry out experiments in an unheated glasshouse. A number of practical improvements were made.

BENTAZONE

For the bentazone experiment all plants were grown on sand, with a day-night temperature of 18-16°C and light-dark period of 12-12 hours, yielding healthy plants. One week after exposure no effects were visible; after two weeks plants treated with the lowest dosage showed red leaves; at higher dosages the leaves became pale. After four weeks the plants were harvested, effects recorded, and the fresh and dry weight of shoot and root measured. The results are shown in Table 3.6 and Figure 3.13.

Table 3.6 Dosages of bentazone and visual effects on *Brassica napus*.

dosage (mg a.i./m²)	effect
144	some yellow colouring
72	some yellow colouring
36	very little yellow colouring
18	very little yellow colouring
3.6	very little yellow colouring
1.44	some red colouring
0	none

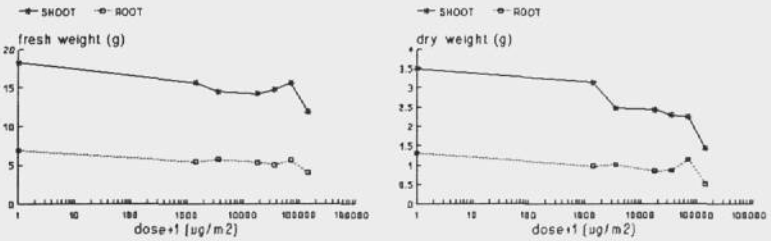


Figure 3.13 Results of glasshouse experiment with bentazone and *Brassica napus*.

There was a significant effect, even at the lowest dosages (Fig. 3.13) (ANOVA, $P < 0.05$). At higher levels, except for the actual field rate, this effect did not increase. Shoot and root weights showed the same tendencies. From the results the ED_{50} values were calculated (Table 3.7). It can be seen that the ED_{50} value is lowest for shoot fresh weight; the ED_{50} value for dry weight is much higher. The ED_{50} for the shoot is somewhat higher than for the root, but of the same order of magnitude.

Table 3.7 ED_{50} values for glasshouse experiment with bentazone and *Brassica napus*.

	shoot ($\mu g/m^2$)	root ($\mu g/m^2$)
fresh	3752	5473
dry	11448	13327

It is concluded that the effect of bentazone becomes visible only after a few weeks, and that these visible effects are in accordance with the biomass effects. At 1 % of the actual field rate a significant effect was found. A preliminary conclusion from this experiment is that the root weight does not yield essentially new information compared with shoot weight. Since dry weight values are much higher than fresh weight values in this case, fresh weight seems to be a more sensitive parameter.

GLYPHOSATE

Table 3.8 and Figure 3.14 show the results of the glasshouse experiment with glyphosate. In the other experiments a high correlation was found between root and shoot weight. In addition, the cleaning of the roots is very time-consuming. Therefore, only shoot weight was measured. Potting soil was used as a substrate.

In this experiment, besides *B. napus*, *P. annua* was used to trace any difference in sensitivity between a dicotyledonous and a monocotyledonous species. Each sample consisted of ten pots with ten oilseed rape plants each and ten pots with 25 grass plants each. The pots were measured as one sample. The effects are shown in Table 3.8 and Figure 3.14.

Table 3.8 Dosages of glyphosate and visual effects on *Brassica napus* and *Poa annua*.

dosage (mg a.i./m^2)	effect on *Brassica napus*	effect on *Poa annua*
216	yellow colouring, dead plants	all plants dead
108	very strongly red-coloured leaves	yellow and dry
54	strongly red-coloured leaves	yellow, epinastic
27	strongly red-coloured leaves	yellow, epinastic
5.4	red-coloured leaves	some yellow colouring, less healthy than untreated
2.16	oldest leaves yellow	none
0	none	none

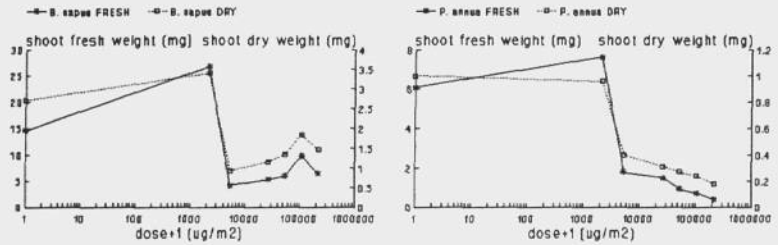


Figure 3.14 Results of glasshouse experiment with glyphosate and *Brassica napus* and *Poa annua*.

The visual effects show a clear relationship with the dosage. This relationship is also found for fresh and dry weight. The biomass at the untreated and the lowest dose differ significantly from the other treatments. Grass plants showed less variation than oilseed rape plants. In the case of *P. annua* differences of approximately 15% could be traced at $P < 0.05$. In the case of oilseed rape even the highest dose did not kill all plants.

Table 3.9 ED_{50} values for glasshouse experiment with glyphosate and *Brassica napus* and *Poa annua*.

	B. napus ($\mu g/m^2$)	*P. annua* ($\mu g/m^2$)
fresh	5338	5374
dry	2827	4277

The ED_{50} values are shown in Table 3.9. For both species and for both fresh and dry weight this parameter is of the same order of magnitude. From this result it can again be concluded that dry weight does not yield new information. Unlike the differences in dose-effect curves, the ED_{50} values of *B. napus* and *P. annua* are very well comparable.

Since in most experiments *B. napus* showed a relatively high variation in biomass, the method was improved, as described in Section 3.2.2.3. This method was tested in the laboratory using MCPA.

MCPA

On the base of deposition measurements in the field, dilutions of 100%, 10%, 2%, 1%, 0.1% and 0% of the recommended field rate were used; the untreated control was treated with water only. One tray with 80 plants was used for each treatment. At the start of the experiment 20 plants were harvested and used to investigate whether there were any differences between the units prior to the start of the experiment. This was not the case. 20 plants were harvested and weighed one, two and three weeks after exposure. The plant condition was recorded at harvesting time. The results are shown in Table 3.10.

Table 3.10 Dosages of MCPA and visual effects on *Brassica napus* after 1, 2 and 3 weeks.

dosage (mg a.i./ m^2)	one week	two weeks	three weeks
100	most plants dead	all plants dead	all plants dead
10	very strongly red-coloured leaves	most plants dead	all plants dead
2	some curling of cotyledons	plants lying down	most plants dead
1	some curling of cotyledons	curling of coty-ledons, epinastic	curling of coty-ledons, epinastic
0.1	none	smaller than untreated	smaller than untreated
0	none	none	none

The effects on biomass are shown in Figure 3.15. The results were tested with ANOVA. After one week a clear effect was found at the highest dosage only. After two weeks there was a clear dose-effect relationship. After three weeks only the lowest dosage did not differ significantly from the untreated unit.

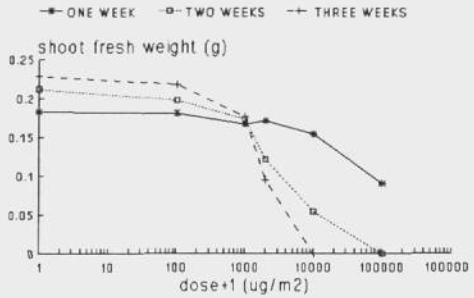


Figure 3.15 Results of laboratory experiment with MCPA and *Brassica napus*.

Table 11 shows the ED_{50} values for MCPA. As is usual in toxicity experiments, the ED_{50} values decrease with time. From the results it can be concluded that the bioassay method can be used for investigating the side-effects of hormone-type compounds, too. Effects were found from 1% of the actual field rate upwards. No evidence was found for a growth-stimulating effect of low dosages. Harvesting at three time intervals gives an opportunity to follow the development of effects with time. The method using more, but younger plants gives clear results and less variation. Differences of 10% in fresh weight could be traced at $P < 0.05$.

Table 3.11 ED_{50} values for glasshouse experiment with MCPA and *Brassica napus*.

	ED_{50} *B. napus* ($\mu g/m^2$)
one week	14929
two weeks	3165
three weeks	1708

Conclusions of glasshouse experiments

The glasshouse experiments show that it is well feasible to conduct the experiments with a larger number of plants. The sensitivity of the test is thus increased. The use of the monocotyledon *P. annua* yields clear results. In the glasshouse experiments effects of bentazone and MCPA were found from 1% of the actual field rate upwards.

3.2.2.4 Experimental plot 1993 and 1994

An overview of the tests performed in the experimental plot is given in Section 3.2.2.3. The results are dealt with per compound tested and summarized in figures showing distance-weight correlations. The untreated control is assumed to be at 80 m distance, for a good fit in the figures. The figures also show the deposition rates. All the results were tested using one-way analysis of variance.

GLYPHOSATE

The first experiment (May 1993) with glyphosate and *B. napus* still had a deviating lay-out (one flower box, small numbers of large plants; see § 3.2.2.3). In addition, a number of circumstances were not ideal. The wind was not in the direction of the bioassays, resulting in low deposition rates at the sampling points. A shortage of water occurred in the non-exposed units, leading to major variation. The results indicate an effect in the 100%-treated unit only. Dry and fresh weight were highly correlated. To reduce the variation and avoid the aforementioned negative effects, the experiment was repeated in October 1993 (Fig. 3.16) and in April 1994 (Fig 3.17) in an improved lay-out.

A clear correlation between distance and effect was observed. In October there was grazing by snails in the untreated and the 16-m units. In 1994 two untreated units were used. The field lay-out was such that the wind was in the direction of the bioassay units.

In 1994 only fresh weight was recorded, because in previous experiments the correlation between dry and fresh weight was very high. Effects were found up to 8 m from the treated plot. In the 1994 experiment, at each distance ten boxes with four plants each were added, 1 m apart, parallel to the plot to be sprayed. The results of these ten boxes, harvested after 14 days, are shown in Figure 3.17. It can be concluded that the results with the separate plants do not differ significantly from one unit with 150 plants.

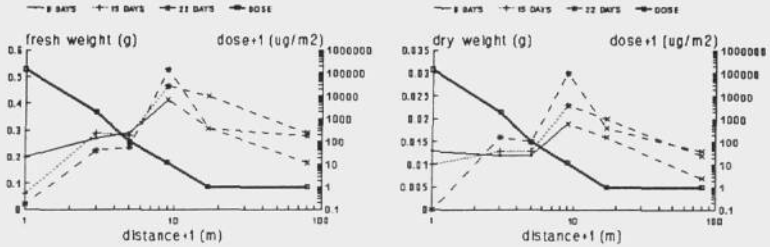


Figure 3.16 Effects of a glyphosate treatment on 06-10-93 on *Brassica napus*; wind speed 3.5 m/s, south; plants at 16 and 80 metres were grazed by snails.

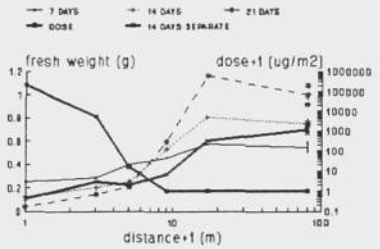


Figure 3.17 Effects of a glyphosate treatment on 12-04-94 on *Brassica napus*; wind speed 6 m/s, north-west.

Table 3.12 $Edist_{50}$ and ED_{50} values for field experiment with glyphosate and *Brassica napus*.

	06-10-93 fresh weight		06-10-93 dry weight		12-04-94 fresh weight	
	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)
one week	4.62	139	8.17	16	5.47	7.0
two weeks	3.28	1044	5.46	65	7.26	2.7
two weeks sep.					9.99	1.5
three weeks	4.32	196	3.59	575	7.92	2.1

Table 3.12 shows the $Edist_{50}$ values and the corresponding dose rates for glyphosate and *B. napus*. In this table it can be seen that in the second experiment the response of *B. napus* to glyphosate is much more sensitive. In the first experiment, however, the 16 m and the untreated units suffered from snail grazing; for modelling the $Edist_{50}$ value these sample points were not taken into account, resulting in an underestimated $Edist_{50}$ value. In the first experiment no clear correlation between time and $Edist_{50}$ values was found; neither were fresh and dry weight clearly correlated. In the 1994 experiment, however,

the $Edist_{50}$ values increased with time and a rather low ED_{50} value was found. The separate boxes in this case were even more sensitive.

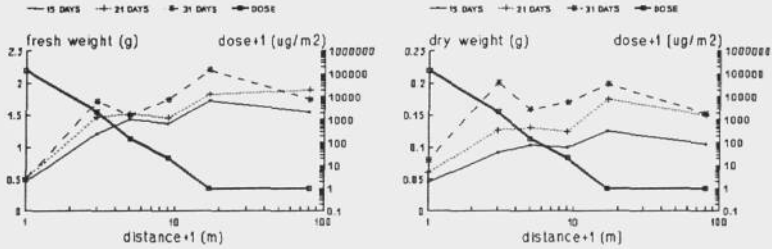


Figure 3.18 Effects of a glyphosate treatment on 01-06-93 on *Pisum sativum*; wind speed 3 m/s, south-west.

In the case of pea *Pisum sativum* (Fig. 3.18) a correlation between distance and biomass was found. In most cases, however, the differences are not statistically significant. In addition, pea plants appeared to be very sensitive to fungi and aphids. In this experiment these factors did not differ between distances.

Table 3.13 $Edist_{50}$ and ED_{50} values for field experiment with glyphosate and *Pisum sativum*.

	fresh weight		dry weight	
	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)
one week	2.45	2439	2.52	2213
two weeks	2.82	1478	2.43	2514
three weeks	1.90	6779	1.01	148630

Table 3.13 shows the $Edist_{50}$ values. In this experiment $Edist_{50}$ seems to decrease with time. This is probably due to the fact that only the treated control suffered a clear effect, and the plants at the other sample points continued to grow. The correlation between fresh and dry weight $Edist_{50}$ values is not clear. Compared to *B. napus*, *P. sativum* appears to be much less sensitive.

Figures 3.19, 3.20 and 3.21 show the results of the experiments with glyphosate and *P. annua*. Most differences are statistically significant. As in the case of *B. napus*, effects were found up to 8 m downwind of the treated plot. In the 1994 experiment separate boxes were also added (Fig. 3.21). In this case it can be seen that at 16 m and in the untreated control in the separate boxes growth is higher than in the multi-compartment trays. In the exposed boxes (up to 8 m) the differences are less clear.

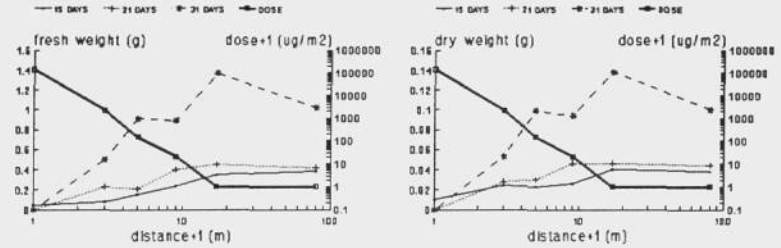


Figure 3.19 Effects of a glyphosate treatment on 01-06-93 on *Poa annua*; wind speed 3 m/s, south-west.

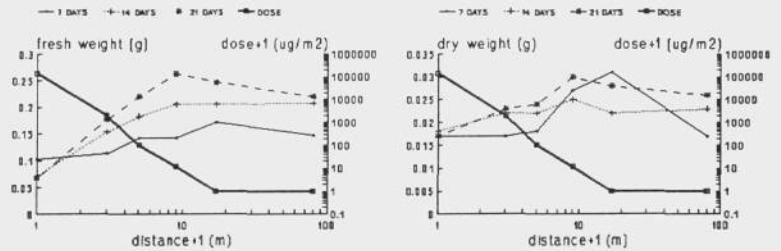


Figure 3.20 Effects of a glyphosate treatment on 06-10-93 on *Poa annua*; wind speed 3.5 m/s, south.

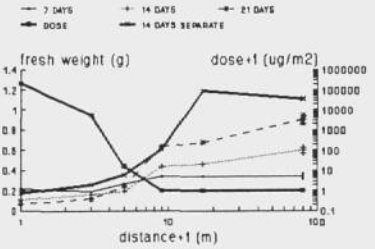


Figure 3.21 Effects of a glyphosate treatment on 12-04-94 on *Poa annua*; wind speed 6 m/s, north-west.

Table 3.14 shows the results of $Edist_{50}$ and ED_{50} values for *P. annua*. The results show a positive correlation between $Edist_{50}$ and time in the 1994 experiment only. Furthermore, the 1994 experiment is much more sensitive than the 1993 experiments. In the 1994 experiment the sensitivity of *P. annua* is comparable with *B. napus*. In the 1994 experiment with *P. annua* the wind speed was 6 m/s. Higher deposition rates were expected in this case. It is not clear why the deposition rates were so low.

Table 3.14 $Edist_{50}$ and ED_{50} values for field experiments with glyphosate and *Poa annua*.

	06-10-93 fresh weight		06-10-93 dry weight		12-04-94 fresh weight	
	$Edist_{50}$ (m)	ED_{50} ($\mu g/m2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)
one week	3.61	552			5.06	14
two weeks	2.73	1329	1.97	1329	5.38	7.6
two weeks sep.					8.65	1.9
three weeks	2.74	1311	2.40	2164	8.07	2.1
01-06-1993						
two week	7.53	34	4.65	202		
three weeks	3.58	861	2.77	3824		
four weeks	3.02	2092	2.95	2429		

DIQUAT

The results of the bioassay experiments with diquat are shown in Figures 3.22 and 3.23.

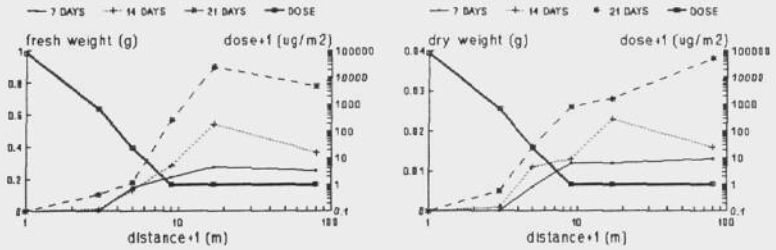


Figure 3.22 Effects of a diquat treatment on 22-07-93 on *Brassica napus*; wind speed 3.5 m/s, west.

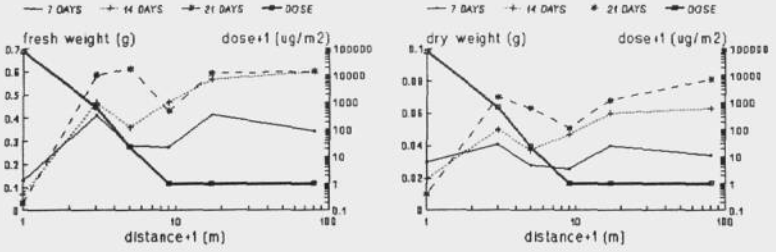


Figure 3.23 Effects of a diquat treatment on 22-07-93 on *Poa annua*; wind speed 3.5 m/s, west.

The results of the experiment with *B. napus* show a correlation between distance and effect. At 8 m, where no deposition was measured, a significant effect was still detectable. *P. annua* shows an effect only in the 100%-treated unit. This is in accordance with the directions for use, in which a remark is made concerning its limited effectiveness for grass (Mandersloot, 1993). Furthermore, diquat is designed to control *B. napus*. The experiment with *Pisum sativum* was totally disturbed by pests and diseases. Therefore, further experiments with *P. sativum* were abandoned.

Table 3.15 $Edist_{50}$ and ED_{50} values for the field experiment with diquat and *Brassica napus*.

	fresh weight		dry weight	
	$Edist_{50}$ (m)	ED_{50} ($\mu g/m2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)
one week	4.84	14.7	5.48	8.4
two weeks	5.48	6.3	4.47	22.9
three weeks	6.69	4.3	6.72	4.3

Table 3.15 shows the 50%-effect distances. For *B. napus* $Edist_{50}$ for fresh weight increases with time. ED_{50} values are rather low. Dry weight results are of the same order of magnitude. For *P. annua* the model used could not be applied in a meaningful way, because the dose-effect relationship is unclear. Nevertheless it can be concluded that *P. annua* is much less sensitive, as can be seen in Figure 3.23.

BENTAZONE

The results of the experiments with bentazone are shown in Figures 3.24, 3.25 and 3.26. From the results it can be concluded that *B. napus* suffers effects up to 16 m from the treated plot. The separate plants do not yield very different results from the standard units. In the case of *P. annua* effects are less clear. Only after one week, a small but clear effect was observed. In the directions for use the insensitivity of *P. annua* to bentazone is mentioned (Mandersloot, 1993). Furthermore, it is intended to control *B. napus* seedlings.

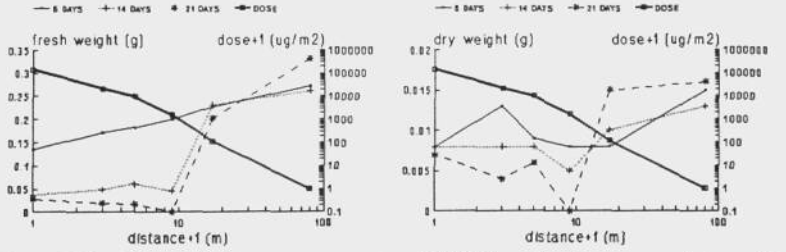


Figure 3.24 Effects of a bentazone treatment on 21-09-93 on *Brassica napus*; wind speed 5 m/s, south-west.

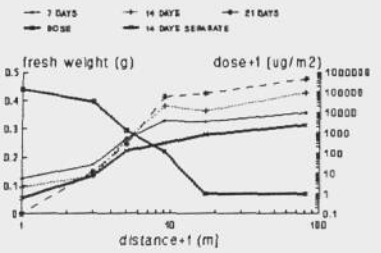


Figure 3.25 Effects of a bentazone treatment on 03-03-94 on *B. napus*; wind speed 5.5 m/s, south-west.

Table 3.16 $Edist_{50}$ and ED_{50} values for the field experiment with bentazone and *B. napus*.

	21-09-93 fresh weight		21-09-93 dry weight		03-03-94 fresh weight	
	$Edist_{50}$ (m)	ED_{50} (μg/m2)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)	$Edist_{50}$ (m)	ED_{50} ($\mu g/m^2$)
one week	9.06	1001	37.78	28.2	4.48	2502
two weeks	13.34	292	32.59	36.7	5.17	1168
two weeks sep.					4.38	2842
three weeks	16.76	156	15.86	180.6	4.53	2353

Table 3.16 shows the 50%-effect distances for *B. napus*. From these results it can be concluded that in the second experiment the response was less sensitive. In the first experiment a very high deposition was found, and therefore the $Edist_{50}$ is found beyond the last bioassay unit at 16 metres. In this case the separate boxes gave a higher ED_{50} value than the multi-compartment trays.

As can be seen in Figure 3.26, *P. annua* is not very sensitive to bentazone; only with the 100%-treated control was an effect found. The model could therefore not be applied to calculate the 50%-effect distance.

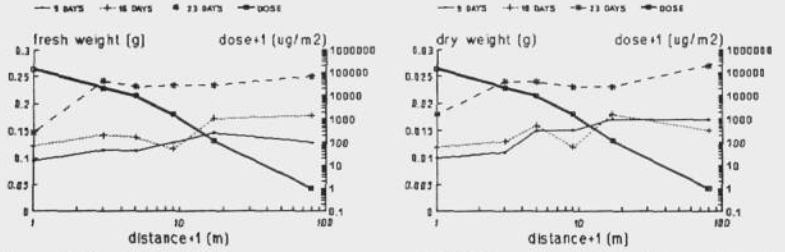


Figure 3.26 Effects of a bentazone treatment on 21-09-93 on *Poa annua*; wind speed 5 m/s, south-west.

3.2.3 Discussion and conclusions

In this study a number of bioassay experiments were conducted with vascular plants. The first, preliminary studies showed that field bioassays can be conducted with *Brassica napus* and *Poa annua*. Some indications for the occurrence of side-effects in the field were obtained in the case of atrazine/bentazone treatment.

The method was then improved using glasshouse experiments and experiments on an experimental plot. Using twenty plants it appeared to be possible to trace differences of 5% in growth reduction with 95% confidence. Using the more fundamental approach of the formula mentioned in Section 3.1, 20-40 plants appears to be necessary to trace differences of 5% of the untreated control. If 10% differences are chosen in all cases, 20 plants are sufficient. It was investigated whether plants influenced one another by comparing the plants at the edges of a unit with those in the middle; no significant differences in weight were found. In addition, in a number of experiments 20 plants from each unit were weighed before the experiment to examine whether there were any differences between the units prior to the experiment. This was not the case.

A number of experiments was conducted on this experimental plot. The results of the ED_{50} values for all experiments are summarized in Table 3.17. The results of the glasshouse experiments are also shown. In the case of diquat and glyphosate, however, there were large differences between the bio-assay units in the glasshouse and field. The most important difference is the age of the plants. In the glasshouse experiment older plants (approx. two months) were used, so that a much higher ED_{50} value was to be expected, as was indeed the case. The plants used in the experimental plot were less than two weeks old.

The inter-species differences in ED_{50} values are as expected. For diquat and bentazone it is known that *P. annua* is less sensitive than *B. Napus*. For glyphosate there are large differences between the two species. For *P. sativum* a rather high ED_{50} value was found.

Table 3.17 Fresh-weight ED_{50} values after three weeks for all experiments with vascular plants; gh = glasshouse experiment.

	diquat µg/m2	bentazone µg/m2	glyphosate µg/m2	MCPA µg/m2
Brassica napus	4.3	3752 gh 156 2353	5338 gh 196* 2.1	1708 gh
Poa annua	> 663	> 21519	5374 gh 1311 861 2.1	
Pisum sativum			6779	

* predation at 16 m and in untreated control

In general, Table 3.17 shows large differences between the replicated experiments. There are many possible reasons for this variation. In the first place there are the varying environmental conditions: temperature and other weather conditions varied among experiments. In addition, growing conditions varied, so that even if plants are of the same age, growth may vary. If results are to be comparable, therefore, it is recommended to carry out this kind of experiment under more controlled conditions. In particular, the growth and effect periods can be controlled by using a climate chamber, thus excluding this source of variation.

The distances at which effects are found of course depend on wind speed. At moderate wind speeds effects are found at about 4 m from the treated plot. Significant effects are regularly found at 8 m, however, and sometimes at the 16 m sampling point. With respect to other studies, the most relevant research work has been carried out in England. Elliot and Wilson (1983) have published a review of the effects of herbicide drift. This study reports on several cases in which crop damage is attributable to drift.

Marrs et al. (1989) have studied the effects of five commonly used herbicides (three of which - glyphosate, MCPA and mecoprop - are also approved for use in the Netherlands) on 15 plant species considered important from a nature-conservation point of view (incl. black knapweed *Centaurea nigra*, yellow archangel *Lamiastrum galeobdolon*, primrose *Primula vulgaris* and betony *Stachys officinalis*). Specimens of these species were placed at various distances from the target plot and the impact of spraying assessed, focusing on the effects of spray drift. Lethal effects were found up to 6 metres away from the target plot. The greatest distance at which effects were still detectable was 20 metres (in self-heal *Prunella vulgaris*). Effects on flowering and seed production were found up to 10 metres away from the plot. In a follow-up study (Marrs et al., 1991a), the impact at shorter distances (up to 4 metres) was investigated. Although visible effects were observed in this study, at the end of the growing season even plants that had been directly sprayed showed no stunting of growth. In another study (Marrs et al., 1991b), however, as a result of drift three species (ragged robin *Lychnis flos-cuculi*, primrose *Primula veris* and buttercup *Ranunculus acris*) showed a reduction in flowering performance; in microcosm experiments the balance between species was affected. Our results are in good agreement with these studies.

In their book 'Herbicide Bioassay', Streibig and Kudsk (1993), give an overview of the use of all kinds of bioassays relating to herbicides. However, there is hardly any focus on field bioassays aimed at investigating the side-effects of pesticides.

Effects could be traced at low deposition rates; sometimes effects were recorded below the detection limit of the deposition measure method. These effects may be due to vapour drift. That vapour drift can play a part has been shown in previous studies (see: De Jong *et al.*, 1992). In England, again, the impact of vapour drift on potted plants has been investigated (Eagle, 1982). For *Brassica napus* effects were observed up to 100 metres away from the sprayed plot in plants placed in the field between 8 and 30 hours post-treatment. Relatively brief exposure to vapour drift was already sufficient to cause damage. A study on the effects of low concentrations of mecoprop on oilseed rape (Breeze and Timms, 1986) indicates that spray drift can be expected to have a deleterious impact up to 10 metres away from the treated plot.

Breeze (p.a. 1988), who has carried out many studies on the effects of drift on vascular plants (agricultural crops), including modelling studies and dose-effect relationships, reports that scarcely any research has focused on sublethal effects in the field. At the moment, there is still insufficient know-how to develop standard field testing methods. However, Breeze (op. cit.) certainly anticipates an impact and considers development of field procedures extremely important.

From the results of our study we conclude that *Brassica napus* and *Poa annua* are very suitable for use in bioassays for the side-effects of pesticides at low deposition rates. The two species show different sensitivities to different compounds. For the compounds studied, however, *P. annua* did not react more sensitively than *B. napus*. *Pisum sativum* could not successfully be used in the bioassay units, because it appeared to be to sensitive to pests and diseases.

The bioassay can also be used to determine the effectiveness of drift-reducing measures, such as buffer zones. Marrs *et al.* (1992) used bioassays with common sorrell *Rumex acetosa* to assess the use of buffer zones for protecting sensitive sites in upland Britain. However, the bioassays can also be used simply as a sensitive method to detect herbicide drift.

3.3 Herbivores

In this section the methods (§ 3.3.1) and results (§ 3.3.2) of the bioassays with herbivores are presented. The results are discussed in § 3.3.3.

3.3.1 Materials and methods

Test species selection

As a test species to study pesticide side-effects on herbivores, the caterpillar of the great white butterfly *Pieris brassicae* was selected. The main reasons were: it is an important herbivorous insect, it is very common in the Netherlands (Tax, 1989), it is known to be relatively sensitive to a number of insecticides (Sinha, 1989) and it is easily reared (Van Halder, 1991). Furthermore, *P. brassicae* is the subject of many studies (Feltwell, 1982) and there is therefore plenty of information about its rearing, its sensitivity to insecticides, the effects to be expected and the effects of disturbing factors. Davis *et al.* (1991a, 1991b, 1993) also successfully used *P. brassicae* larvae in field experiments.

In 1992, a number of preliminary experiments with larvae of *P. brassicae* (L.) were conducted under practical agricultural conditions. Based on these results, the test with *P. brassicae* was developed under more controlled conditions in 1993 and 1994.

3.3.1.1 Experiments 1992

In 1992, *P. brassicae* was used on Chinese cabbage *Brassica oleracea* and oilseed rape *B. napus* as substrates. Samples consisted of two potted plants; the number of caterpillars varied between two and eight, depending on the quantity of food plant at the start of the experiment (Table 3.18).

Table 3.18 Experiments with *Pieris brassicae*.

crop/pesticide	number of units	caterpillars/plant
HERBICIDES		
maize/atrazine,bentazone	10	2
FUNGICIDES		
potato/maneb	10	4
potato/maneb	4	8
fruit/captan	10	8
fruit/captan	4	6
INSECTICIDES		
tree nursery/acephate	10	4

Caterpillars were 1-2 weeks of age. The plants were covered with 1-mm gauze. After one week the bioassay units were taken into the laboratory and survival was measured; caterpillars were followed until metamorphosis and the number of butterflies were counted. The results were tested using a Kruskal-Wallis analysis of variance.

3.3.1.2 Experiments 1993 and 1994

Insect culturing

A culture of *P. brassicae* was established from egg batches obtained from the Dutch Butterfly Foundation, Wageningen. Egg batches were placed on potted cabbage plants. Larvae were reared in the laboratory at 21°C and were transferred to new cabbage plants when required. When no cabbage plants were available, commercial, organically grown cabbage was provided, not treated with pesticides. First instar larvae (48-72 hours of age) were used, these being most susceptible (Grosscurt, 1977; Sinha *et al.*, 1990). In a few cases the eggs were stored at 4°C for a maximum of three days, in order to gather the quantity of first instar larvae needed at the moment of the experiment.

Bioassay method

The method was first tested in a glasshouse under controlled conditions. Larvae 48-72 hours old were placed on whole oilseed rape *Brassica napus* plants and exposed to the test compound. After exposure the plants were covered with 1-mm gauze. Subsequently, the bioassay units were brought into the field to test the applicability in a field experiment. In this field trial Chinese cabbage *Brassica oleracea* was used as a host plant. Because whole plants were used, the effects could be studied until emergence.

Compounds

The experiments were conducted with pirimicarb (Dimilin®) and diflubenzuron (AA Fleur®). Pirimicarb is known to have a relatively low toxicity for *P. brassicae*, while diflubenzuron is intended to control caterpillars (Sinha *et al.*, 1990, Mandersloot, 1993). Pirimicarb has a choline-esterase-inhibiting mode of action and has a systemic activity. Diflubenzuron acts by inhibiting chitin synthesis, and therefore it interferes with the formation of the cuticle; thus effects become visible at the next moult (Feltwell, 1982).

Experiments

With pirimicarb, indoor experiments in petri dishes and on whole plants were conducted. The petri-dish experiment was carried out to obtain an indication of the sensitivity range of the larvae for pirimicarb. In the whole-plant experiments effects could be investigated over a longer period of time (until emergence). The whole-plant experiment was conducted in a glasshouse to test the suitability of the bioassay units under controlled conditions. Due to lack of time and bad weather conditions no field experiment could be conducted with pirimicarb.

The petri-dish experiment with pirimicarb was conducted applying a single topical dose of 0.2 μl to each of ten larvae in a petri dish using a micro-pipet. The applied rates were 100%, 10%, 2%, 1% and 0% of the actual commercial application rate of 0.42 g/l pirimicarb (Mandersloot, 1993), using five petri dishes per treatment. Mortality was recorded after one day.

In the whole-plant experiment ten larvae were placed on seven *B. napus* plants each per treatment. The plants and larvae were exposed by means of a plant sprayer. Exposure was based on the actual rate of 0.25 kg/ha. Based on the surface of the pots an application of 0.38 mg/plant pirimicarb was calculated for the actual rate. The same rates as in the petri dish were used (100%, 10%, 2%, 1% and 0%). Mortality was recorded until emergence; percentage of pupated larvae after 34 days and pupa weight were used as sublethal effect parameters.

With diflubenzuron a petri-dish experiment in the laboratory, a whole-plant experiment in the glasshouse and two experiments in the field on an experimental plot were conducted. The petri-dish experiment was conducted applying a single topical dose of 0.2 μl to each of ten larvae in a petri dish using a micro-pipet. The applied rates were 100%, 10%, 2%, 1% and 0% of the actual commercial application rate of 0.24 g/l diflubenzuron (Mandersloot, 1993), using one petri dish per treatment. Mortality was recorded after five days. In the glasshouse experiment six larvae per plant and five plants per treatment were used. The actual field rate is 96 g/ha, equivalent to a dose of 0.16 mg per plant. The same rates were used as with pirimicarb. Effects were followed until emergence.

In the field experiments a 5x10-m experimental plot (Fig. 3.1) was sprayed with a knapsack sprayer at a rate of 100 g/ha. The samples consisted of five potted plants with six larvae each in the first experiment (1993), and eight potted plants with ten larvae each in the second experiment (1994). The sample points were situated 0, 2, 4, 8 and 16 m downwind of the treated plot; one sample remained untreated, serving as a control. The wind speed at the time of spraying was 3.5 m/s in 1993 and 4.5 m/s in 1994. Deposition was measured using water-sensitive paper (*cf.* De Snoo & De Wit, 1993). After spraying, the bioassay units were taken back to the laboratory to study the effects until emergence.

Statistical analysis

The results were tested using a Kruskal-Wallis analysis of variance to detect any differences in mortality between the exposure rates. A significant result indicates that results from at least one treatment differs from one other treatment. If the test result was significant, a pairwise test (Mann-Whitney-U) was used to determine which exposure rates differed significantly (Siegel, 1988). The differences in pupa weight in the pirimicarb whole-plant experiment were tested using a one-way analysis of variance (Sokal & Rolf, 1981). LD_{50} values were estimated using the following logistic model, calculating the mortality percentage (M):

$$M = (M_{max}-M_{untr})/(1+ae^{-b\mathrm{LOGD}})+M_{untr}$$

in which M_{max} is the maximum response (100% mortality), M_{untr} is the mortality in the untreated control units, a and b are constants, and D is the dose. Using a Quattro-Pro spreadsheet a best fit of the model to the data was estimated, and the LD_{50} value was calculated from the resulting model. For the field trials the model was applied with distance instead of dose, and the dose calculated from the distance-deposition relationship (see Section 3.2.1.3).

3.3.2 Results

3.3.2.1 Results 1992

The results of the experiments in 1992 are shown in Figures 3.27 to 3.30.

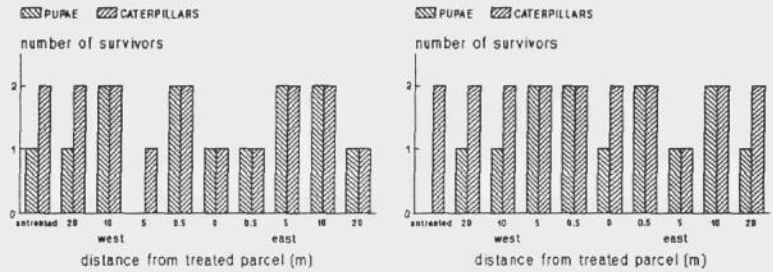


Figure 3.27 *Effects of two atrazine/bentazone treatments in maize 03-06-92 on Pieris brassicae*; wind speed 3 m/s, West.

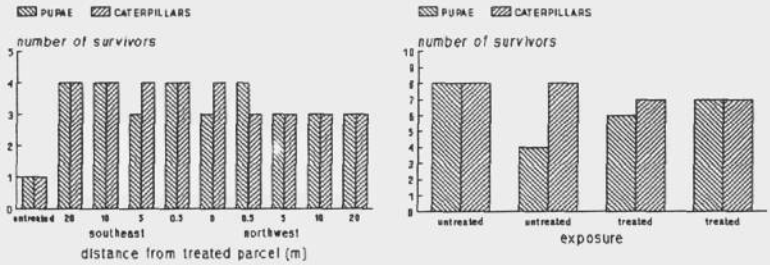


Figure 3.28 Effects of two maneb treatments in potato 06-07-92 (wind speed 8 m/s, North-East) and 11-07-92 (wind speed 3 m/s) on *Pieris brassicae*.

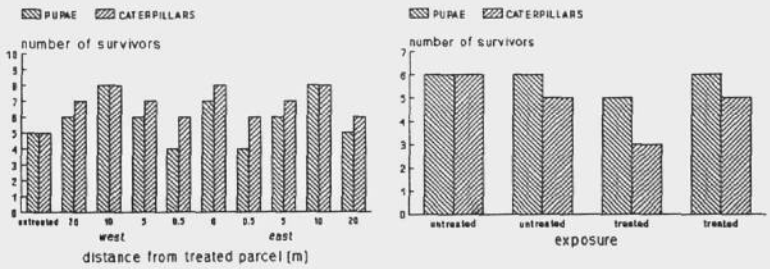


Figure 3.29 Effects of two captan treatments in fruit 09-07-92 (wind speed < 2 m/s) and 27-08-92 (wind speed 5 m/s) on *Pieris brassicae*.

The results show that the herbicide treatment caused no effects. Although there was a visible effect on the food plants, the larvae did pupate. In the case of fungicides no effects were found either.

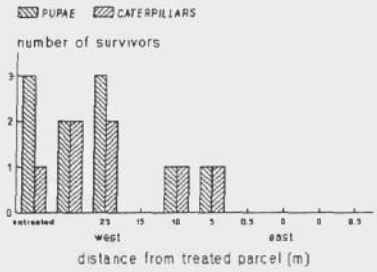


Figure 3.30 Effects of acephate treatment in tree nursery 08-08-92 on *Pieris brassicae*; wind speed 2 m/s.

In only one case was an insecticide used, with a clear effect on the directly exposed samples. Up to 15 m from the treated plot many larvae died. However, in the non-treated situation some larvae also died or escaped.

From the preliminary field experiments it was concluded that caterpillars of *P. brassicae* can be used in bioassays; indications of insecticide side-effects were also obtained outside the treated plot.

3.3.2.2 Results 1993 and 1994

PIRIMICARB

The results of the petri-dish experiment with pirimicarb are shown in Figure 3.31. Mortality was recorded one day after treatment; most caterpillars died in the first few hours, however. An LD_{50} value of 503 $\mu g/m^2$ (2% of the actual rate) was found.

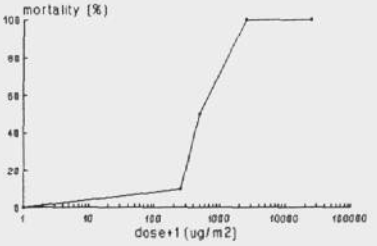


Figure 3.31 Dose-mortality curve of laboratory experiment with pirimicarb 24 hours post-treatment.

Figure 3.32 shows the results of the whole-plant experiment with pirimicarb. In this experiment some larvae 'vanished'. Two possibilities exist: i) larvae escaped from the unit, or ii) dead larvae were not found. Since the very young larvae, in particular, are very small (approx. 5 mm), it is well possible that they had decomposed or were not visible on the soil or between the leaves. In the analyses of the results the total number of larvae was corrected for the number of larvae that had 'vanished', because there was no reason to assume whether they were dead or alive.

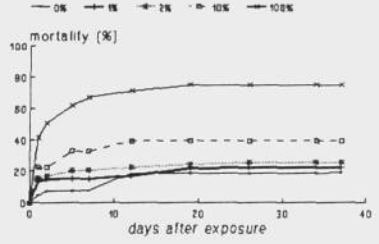


Figure 3.32a Time-mortality curve for glasshouse experiment with pirimicarb.

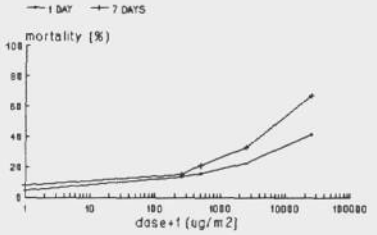


Figure 3.32b Dose-mortality curve at one and seven days post-treatment.

Figure 3.32a shows that most larvae died within one day of treatment; subsequently, only at the highest rates was there a further significant mortality, indicating an acute effect only. There were significant differences in mortality between the treatments (Kruskal-Wallis, $P < 0.01$). The untreated units differed significantly from all the others (Mann-Whitney-U, $P < 0.05$) except for the lowest rate: 1%. At the other rates the only significant difference in mortality was between the 100%-treated units and the 1%- and 2%-treated units (Mann-Whitney-U, $P < 0.05$). Even at the actual field dose there was no 100% mortality. After one day the $LD_{50} > 100\%$ (25000 $\mu g/m^2$) of the actual field dose (Fig. 3.32b). After seven days the LD_{50} was 8751 $\mu g/m^2$ (35% of the actual field dose).

Table 3.19 Total number of surviving pupae (pro-pupae and pupae) as a percentage of total number of surviving larvae (mean ± standard deviation) and pupa weight 34 days after pirimicarb treatment in a whole-plant glasshouse experiment.

% of actual rate	pupae (%) 34 days	number of plants[1]	pupa weight (mg)	total number of pupae
0	44 ± 34	6	328 ± 55	38
1	34 ± 31	5	329 ± 57	33
2	64 ± 44	4	329 ± 54	29
10	28 ± 24	4	314 ± 57	27
100	-	0	360 ± 25	13

[1] Only plants with ≥ 5 living individuals were taken into account.

Effects on growth and development were also studied. As a development parameter the number of pupae after 34 days was estimated as a percentage of the total number of surviving larvae. A distinction was made between real pupae and pro-pupae (larvae attached, but not yet encapsulated). Growth was measured using pupa weight. The results are shown in Table 3.19. For pupae, pro-pupae and total number of pupating and pupated individuals, no significant differences were found between treatments after 34 days. Neither was there any significant difference in pupa weight between treatments. All pupae emerged.

DIFLUBENZURON

In the petri-dish experiment with diflubenzuron only the caterpillars in the untreated petri dish survived. In all the other treatments, from 1% of the actual rate (96 $\mu g/m^2$) upwards, all caterpillars had died five days after exposure. From this result it can be concluded that the LC_{50} value is less than 96 $\mu g/m^2$. Mortality was recorded one, four and five days after treatment. In line with the mode of action, the caterpillars died at moulting, but only after a few days.

The results of the glasshouse experiment with diflubenzuron are presented in Figures 3.33a and 3.33b. Figure 3.33a shows that significant differences occur after one day (Kruskal-Wallis, $P < 0.01$), but only at 10% of the actual rate was the mortality significantly higher than in the other treatments (Mann-Whitney-U, $P < 0.05$). After 6 days there was a significant difference in mortality between 2% and lower doses, and between 10% and 100% and lower doses (Mann-Whitney-U, $P < 0.05$). The LD_{50} value after fourteen days is 206 $\mu g/m^2$ (2% of the actual rate) (Fig. 3.33b).

For the sublethal effects only untreated and 2%-treated units were compared, because at 100% and 10% no individuals remained alive (Fig. 3.33a) and the 1%-treated unit did not differ from the untreated unit. After 22 days a clear effect was visible: in the untreated unit 50% of the individuals was pupating or had pupated, and in the 2%-treated unit only 12.5%. After 26 days this difference was even larger: 100% of the untreated individuals had pupated and in the 2%-treated unit only 43%.

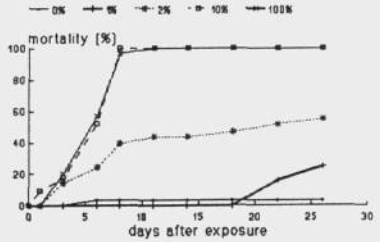


Figure 3.33a Time-mortality curve for glasshouse experiment with diflubenzuron.

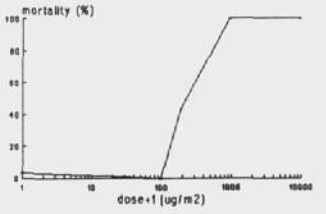


Figure 3.33b Dose-mortality curve at 14 days post-treatment.

Figures 3.34a and 3.34b show the results of the field trial with diflubenzuron in 1993. High mortality was found at lower rates than in the glasshouse experiment (field: after sixteen days $LD_{50} \approx 13\ \mu g/m^2$, 6.9 m from the treated plot; see Fig. 3.34b). Mortality was also found in the untreated unit. Figure 3.34a shows that at 0 and 2 metres mortality was 100% after five days. These treatments differ significantly from the others (Kruskal-Wallis, $P < 0.01$, Mann-Whitney-U, $P < 0.05$). From five days onwards this situation only changed significantly for the 4 m unit. After 9 days no significant difference was found between the 4 m unit and the treated and 2 m units, while the difference between the 2 m unit and the 8 m, 16 m and untreated units became significant (Mann-Whitney-U, $P < 0.05$).

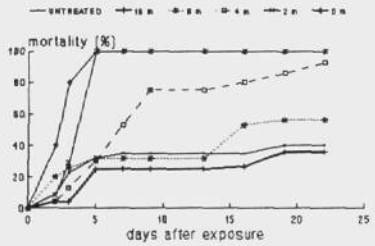


Figure 3.34a Time-mortality curve for field experiment with diflubenzuron.

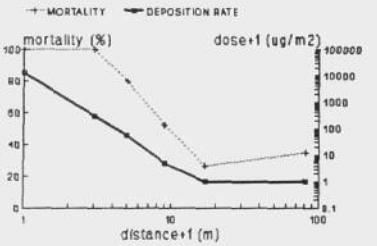


Figure 3.34b Dose-mortality curve at 16 days post-treatment.

Because of the high overall mortality, the number of individuals was too small to analyse the data per plant. All individuals were therefore lumped together per distance. These results are shown in Table 3.20. The results show that after 19 days the number of pupae at 8 m was clearly lower than at 16 m and in the untreated unit. After 22 days this difference had become smaller. The pupa weight after 26 days did not differ significantly among treatments. All pupae emerged.

Table 3.20 Pupae and pupae + pro-pupae as a percentage of total number of surviving individuals 19 days after a diflubenzuron treatment and pupa weight (mean ± standard deviation) after 26 days in whole-plant field experiment.

distance	pupae % (19 days)	pro pupae + pupae % (19 days)	pupa weight (mg) (26 days)	n
untreated	42	83	278 ± 53	11
16 m	50	70	294 ± 48	9
8 m	29	57	286 ± 73	6

The results of the 1994 experiment are shown in Figures 3.34a and 3.34b. The results again show high mortality at low deposition rates. After 4 days there was a significant

difference between untreated units and the sampling points up to 8 metres from the treated plot. Up to three weeks post-treatment the difference between the sampling point at 8 metres from the treated plot and the closer points is also significant. At the end of the period, however, all the caterpillars at the 8 metre point had also died. The only significant differences remaining were those between the untreated and 16 metre samples on the one hand, and the other sampling points at the other hand. The LD_{50} value was lower than in the 1993 experiment (3.2 $\mu g/m^2$, 12.3 m).

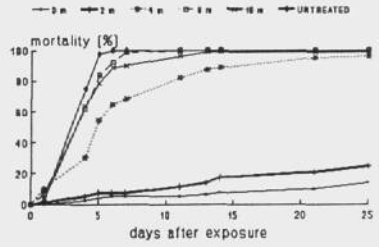


Figure 3.34a Time-effect curve for 1994 field experiment with diflubenzuron.

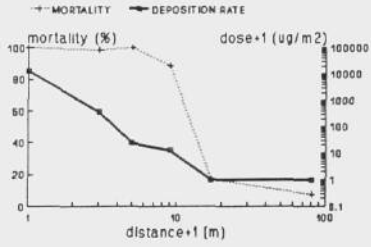


Figure 3.34b Dose-effect curve at 16 days post-treatment.

In this experiment survival in the untreated units was high. As a result, plants were completely consumed, leading to starvation of the caterpillars in the untreated and 16 metre units. From the moment that no food was left, these units were excluded from further analysis. Pupation could therefore not be studied in this experiment. In addition, in all the other units all the caterpillars died, so no comparison with treated units was possible.

3.3.4 Discussion and conclusions

Table 3.21 summarizes the LD_{50} values for the various different experiments. This table shows that in the petri-dish experiment with pirimicarb effects were found at much lower dosages than in the whole-plant experiment. This difference was probably due to a different exposure level. In the whole-plant experiment, most larvae were underneath the leaves during application, so these larvae were not directly exposed. The number of living larvae on each side of the leaves was counted separately. Because of the small numbers of larvae on the upper side, however, no comparison could be made between the two sides of the leaves. It can be concluded, though, that pirimicarb, which is generally presumed to have low toxicity for non-target organisms, in the case of *P. brassicae* already leads to significant mortality at 2% of the actual dose (500 $\mu g/m^2$).

Table 3.21 shows that the LD_{50} for diflubenzuron is lower than for pirimicarb, indicating that with diflubenzuron effects are to be expected at greater distances from the treated plot. When the results of both compounds are compared, it can be seen that pirimicarb causes mortality in the short-term, while diflubenzuron causes slower mortality, but at

lower dosages. These effects are in full agreement with the different modes of action of the two compounds. Only in the case of diflubenzuron was an effect on the duration of the larval stages found.

Table 3.21 LD_{50} values of experiments with *Pieris brassicae*.

	pirimicarb $\mu g/m^2$	diflubenzuron $\mu g/m^2$
laboratory	504	< 96
glasshouse	8751	186
experimental plot		13.3 3.2

Table 3.21 further shows that in the field experiment with diflubenzuron the LD_{50} values are much lower than in the glasshouse experiment. The field circumstances possibly made the larvae more susceptible to the effects of the insecticide. In the first field experiment a relatively large number of larvae 'vanished'. This may have been due to the fact that the units remained uncovered with gauze during and after spraying for two hours. In a control experiment in the field, after two days all larvae on uncovered (untreated) plants had disappeared, while the larvae on gauze-covered plants were still present. This indicates that larvae disappear by falling off the plants owing to weather conditions, or by being eaten. In the 1994 experiment a 1-mm gauze was used, and almost no caterpillars disappeared.

The disappearances might also have been due to a pesticide effect, however. Blackwell (1988) showed that caterpillars became excited and fell off the plants when exposed to the insecticide chlordimeform. This effect was not observed using pirimicarb or diflubenzuron; nevertheless, it is possible that some of the very young larvae fell off the plants as a result of exposure. In that case it is conceivable that the very small larvae could not find the host plant and died on the soil surface of the pots.

Sinha *et al.* (1990) and Davis *et al.* (1991a, 1991b, 1993) also conducted field experiments with diflubenzuron; their results are highly comparable with ours. Contrary to the experiments of Davis we used whole plants, and the effects were recorded until emergence. Sinha and Davis investigated effects at 72 hours post-treatment. Our results (Figs. 33 and 34) show that at 4 m mortality increased significantly between 5 and 10 days post-treatment, indicating that a longer recording period yields important supplementary information.

Davis *et al.* (1991b) show that results for *P. brassicae* can be extrapolated to other species of Lepidoptera living in habitats adjacent to crops, and that *P. brassicae* is relatively sensitive. Therefore, by using *P. brassicae* as a test organism for the side-effects of pesticides, most other Lepidoptera species will be protected.

It can be concluded that *P. brassicae* larvae are suitable for bioassay research and for tracing the effects at low exposure rates outside the target area. Being readily observable, mortality appears to be the most suitable effect parameter.

Davis *et al.* (1991a) also conducted the bioassays at various wind speeds. As expected, a correlation was found between wind speed and drift. In some cases effects were found below deposition levels detectable on the water-sensitive paper.

In another experiment Davis *et al.* 1993 investigated the effects of buffer zones and hedgerows on pesticide drift and mortality of *P. Brassicae*. Results showed effects up to 24 m from the treated parcel. Buffer zones and hedgerows did diminish the distance at which effects occur, but a field margin of 3 or 6 m, as currently being discussed in the Netherlands (De Snoo, 1994), will not prevent mortality of Lepidoptera larvae outside the parcel, however.

3.4 Decomposers

3.4.1 Materials and methods

Test species selection

Many organisms contribute to the decomposition process. A number of invertebrate species contribute to the fragmentation of organic matter, and fungi and bacteria decompose this organic matter into nutrients and minerals.

Earthworms contribute substantially to the fragmentation of organic matter. Many studies have been carried out with this organism (*cf.* van Gestel, 1991). De Jong *et al.* (1990) have developed a guideline for a field trial with earthworms. Bioassays have been conducted by Ebing *et al.* (1984) and appeared to be very suitable for use in field trials. Because extended guidelines exist in Germany (BBA), this organism was not chosen as a test species in this study.

Soil fungi play a dominant part in the decomposition process. Many studies are concerned with the effects of pesticides on mycorrhiza-forming fungi (Menge, 1982; Trappe, *et al.*, 1984; *cf.* de Jong *et al.*, 1992). However, almost all of these studies deal with effects inside the target area, and concern the mycorrhizal fungi on the crop. In these cases it is the degree of root infection after a pesticide treatment that is assessed. Field bioassays with mycorrhizal fungi have not been found in the literature. Laboratory studies appeared to be poorly representative for the field situation (Unestam *et al.*, 1989). Termorshuizen (Wageningen Agricultural University, LUW) suggests the use of leek *Allium porrum* in the field. This plant can then be used to detect the number of mycorrhizal fungi in the soil. However, this method is likely to involve many difficulties. Firstly, detection of mycorrhizal fungi is a technical problem that is rather time-consuming; secondly, the number of mycorrhizal fungi and their dispersal pattern in different types of soil in the Netherlands is not well documented. Therefore, the chance of successfully developing a test with this organism was deemed too small.

Another possibility is the use of mushroom-type fungi. Mushrooms consist mainly of water, however, and studying the effects would constitute mainly a study of their capacity to take up water. Studies of effects on the mycelium are not known, and no methods were found in the literature. Consequently, this type of species was not chosen.

Another possibility is to measure decomposition itself. Two methods are worthy of mention: the use of cotton strips and the use of litterbags. Both methods are described extensively in the literature (Harrisson *et al.*, 1988; Heath *et al.*, 1964, 1966). In the cotton-strip method the loss of tensile strength is assessed and in the litterbag method the decomposition of organic matter is measured. Both methods seem to be suitable. Arbitrarily, we have chosen the litterbag method.

3.4.1.1 Preliminary experiments 1991

Locations and treatments

The experiments were conducted on a tree-nursery parcel and at a bulb-growing parcel. Part of the parcel was disinfected at the end of May 1991 using metham-sodium; the

bulb-growing parcel was disinfected on August 17 using dichloropropene (Fig. 3.35). At both locations effects were studied using litterbags.

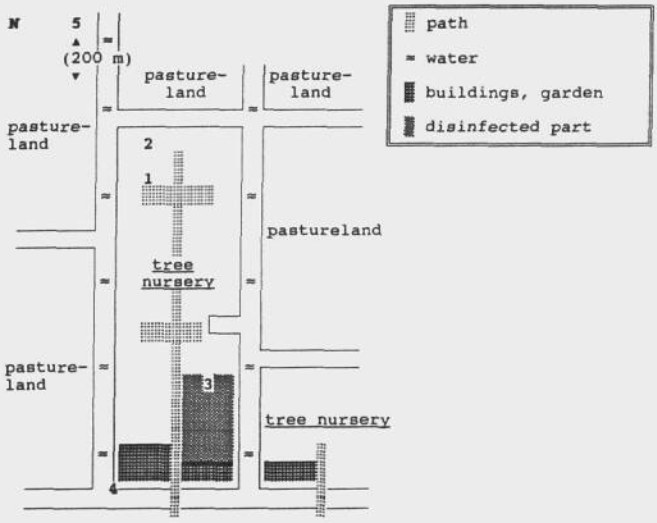


Figure 3.35a Tree-nursery parcel; numbers indicate sampling points; 5 = untreated.

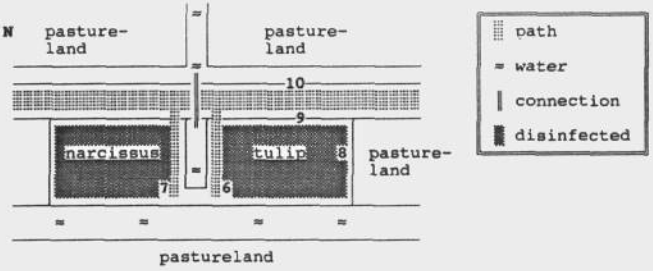


Figure 3.35b Bulb-growing parcel; numbers indicate sampling points; 9 and 10 = untreated.

Bioassay

Heath *et al.* (1964, 1966) investigated the decomposition of several kinds of leaves in litterbags. They used 20 leaf discs of about 2.5 cm per litterbag. Litterbags consisted of 10x7-cm nylon bags of 3-μm mesh. We duplicated the method, using 20x20-cm litterbags with 40-μm mesh. Using this mesh, invertebrates are excluded and decomposition will be caused by micro-organisms only.

In 1991 at each sampling point two litterbags were used and covered with 0.5 cm of soil. In each litterbag 20 discs (cellulose paper, about 2.5 cm) were used as a substrate. Cellulose was chosen because it was assumed to decompose quickly, is uniform and readily available. Litterbags were placed in treated parts of the parcel, in untreated parts and outside the parcel (Fig. 3.35). Litterbags were placed on September 3 and effects on decomposition were studied on November 21.

Degradation of the cellulose paper discs was assessed visually by estimating the remaining disc area after decomposition; the dry weight was also measured. Visual assessment of degradation of each cellulose disc was on the basis of the following classes: 0.5 = some decomposition and 1 to 10 = 10 to 100% decomposition of the total area of a leaf disc, respectively. Dry weight was measured per four discs.

3.4.1.2 Glasshouse experiments

In the winter period some glasshouse experiments were conducted. These experiments had two aims: i) to answer a number of methodic questions, and ii) to validate the field results. Litterbags were placed on the soil in the glasshouse and were covered with 1 cm of potting compost. They were exposed with a plant sprayer. The amount of water and the amount of pesticide were calculated from the recommended field dosages. Untreated controls were treated with water only.

3.4.1.3 Field experiments 1992

In 1992 twenty leaf discs of Chinese cabbage *Brassica oleracea* were used in each litterbag, and dried for three hours at 100°C. Cabbage was obtained from a commercial organic farm to ensure the absence of pesticide residues. For one experiment one cabbage plant was used. Compared with cellulose paper these leaf discs have the advantage of being more natural and decomposing faster (Heath *et al.*, 1964). The fresh weight of each leaf disc was measured before the experiment and a correlation between fresh and dry weight was obtained using a control group of twenty discs.

In the field the litterbags were covered with 1 cm of potting compost. After one week the dry weights of the leaf discs were determined. Litterbags were placed in a treated plot, at 5, 10, 20 and > 400 m distance from the treated plot (Fig. 3.1). Treated plots were agricultural fields: potatoes, treated with maneb (3 kg a.i./ha); fruit, treated with captan (1.5 kg a.i./ha) and a tree nursery, treated with parathion-methyl (0.24 kg a.i./ha) and acephate (1.0 kg a.i./ha). In one case the concentration of captan in the soil covering the litterbags was measured 24 hours after application. This concentration was measured in 395 gram soil on a 37.5x20 cm surface. The results were analysed by ANOVA.

3.4.1.4 Experimental plot 1993 & 1994

Litterbags

In 1993 and 1994 the experiments were conducted on the experimental plot (see Fig. 3.2).

Besides validating the field results, these studies were also set up to improve the sensitivity of the test, for instance by using fresh leaf material, by changing the time of exposure and using other soil types to cover the litterbags.

For validation purposes, in 1993 one experiment with captan and maneb was conducted and in 1994 two experiments with captan. The field dose of 1.5 kg captan in 300 l water per hectare and 3 kg maneb in 400 l water per hectare was used. For the test plot of 5 x 10 m 7.5 g captan in 1.5 l water and 15 g maneb in 2 l water were used. Deposition of drift was measured using water-sensitive paper. Results were also validated in glasshouse experiments. The results were statistically analysed by ANOVA.

Fungi

Parallel to the litterbag research, preliminary experiments were conducted with the fungi responsible for the decomposition. The fungi were cultivated on malt-agar (pH 5.2), which is selective for fungi. Standard methods of diluting and counting were used. No determination of fungi was carried out, but the total number of colonies present in the several samples were counted. Three types of experiments were carried out:

1. An attempt was made to establish a correlation between decomposition and fungi on the leaf discs and between fungicides and fungi on the leaf discs. To this end, extra litterbags with four discs were used to determine the level of infection of the leaf discs. The litterbags were placed in the field just like the others. After one week the leaf discs were put in 250 ml sterilized 0.85% NaCl solution and shaken for one day. After this day a series of dilutions of this solution was made in agar and colonies were counted daily. On dishes with 30 - 300 colonies the number of colonies was counted, and from the counted colonies the number of fungi in the undiluted sample was calculated.
2. A second way to establish a correlation between the level of fungicides and the soil fungi was via the use of the soil itself. For this purpose, homogenized soil was placed in petri dishes and exposed to the fungicide at the same distances from the treated plot as the litterbags. After treatment the soil was treated the same way as the leaf discs described under 1.
3. For the last type of experiment agar plates were used in which an initial population of soil fungi was established. This initial population was obtained from 1 mg soil in 250 ml of the 0.85% NaCl solution. Of this solution, 1 ml was used as the start of a dilution series on agar plates. These plates were exposed at the same distances as the litterbags. Petri dishes were opened shortly before application and closed directly afterwards; unexposed and uninfected plates were used to establish the amount of fungi coming from the air.

3.4.2 Results

3.4.2.1 Preliminary experiments 1991

Tree nursery

Litterbags were harvested after eight weeks of decomposition. Unfortunately, the litterbag in the treated part was lost due to soil tillage. Table 3.22 shows the decomposition percentages in the litterbags that were found. The highest decomposition was found at

sampling point 2, within the parcel; this litterbag was not exposed, however. Decomposition at the untreated sampling point 5 was the lowest. Since none of the remaining sample points were exposed to fungicides, no conclusion about effects on decomposition can be drawn. The visual estimates seemed to indicate less decomposition. This can be explained by the fact that the area of the disc was estimated, and discs can become thinner without this being reflected in the disc area.

Table 3.22 Mean and standard deviation of decomposition in tree nursery; location numbers refer to Fig. 3.35a; means followed by the same letter in each column do not differ significantly at 5% level according to Student-Newman-Keuls (SNK) multiple range test.

location number	decomposition (%); mean ± s.d.	
	dry weight (n=10)	disc area (n=40)
1	70b ± 8	14a ± 12
2	98c ± 1	30b ± 25
5	53a ± 10	6a ± 2

Bulb-growing parcel

Table 3.23 shows the results of the decomposition in the bulb-growing parcel. Decomposition was found to be highest at the untreated sampling points (9 and 10). For dry weight, the decomposition within the disinfected parts of the parcel was lower than at the untreated sampling points. The visual method again resulted in less clear differences.

Table 3.23 Mean and standard deviation of decomposition in bulb parcel; location numbers refer to Fig. 3.35b; means followed by same letter in each column do not differ significantly at 5% level according to SNK multiple range test.

location number	decomposition (%); mean ± s.d.	
	dry weight (n=10)	disc area (n=40)
6	75b ± 10	34b ± 25
8	40a ± 4 (n=5)	9a ± 4 (n=20)
9 (untreated)	99c ± 0	35b ± 16
10 (untreated)	94c ± 7	43b ± 28

Conclusion

From the preliminary experiments it was concluded that the test method can be developed further. The visual method will not be used any more because of the poorer results.

3.4.2.2 Field experiments 1992

In 1992 field experiments were conducted with insecticides, herbicides and fungicides.

The results of four experiments in an insecticide-treated tree nursery parcel are shown in Table 3.24. In the cases of acephate and parathion treatments effects were found. Similar effects were also found at a time there was no exposure, however. Therefore, it is not certain that differences were caused by the insecticide treatment. Furthermore, there was a large variation between the litterbags undergoing the same treatment, i.e. in the same parcel.

Table 3.24 Decomposition after exposure to acephate and parathion in tree nursery; means followed by same letter in each column do not differ significantly at 5% level according to SNK multiple range test.

treatment		decomposition (%)
I.	untreated	56b ± 14
	inside parcel	24a ± 7
II.	untreated	59a ± 11
	untreated	60a ± 15
	1 kg/ha acephate	56a ± 12
	1 kg/ha acephate	70b ± 9
III.	untreated	80b ± 4
	untreated	80b ± 6
	inside parcel	77b ± 6
	inside parcel	72a ± 5
	inside parcel	79b ± 5
	inside parcel	77b ± 3
IV.	untreated	69 b ± 5
	untreated	67ab ± 4
	240 g parathion/ha	65a ± 7
	240 g parathion/ha	65a ± 5

In one case effects of a herbicide (Roundup, glyphosate) treatment were studied. The results (Table 3.25) show no significant differences between treated and untreated sampling points.

Table 3.25 Decomposition after exposure to glyphosate in a bulb parcel; ANOVA: $P > 0.05$.

treatment	decomposition (%) ± s.d.
untreated	89 ± 2
untreated	91 ± 3
2160 g glyphosate/ha	89 ± 2
2160 g glyphosate/ha	90 ± 4

With fungicide treatment effects were found in a number of cases. A number of experiments yielded no results, because of differences in circumstances: in the first experiment

some litterbags were placed before, and others after treatment. For reasons of haste a number of litterbags were placed on the (grass) vegetation instead of the soil. In addition, during this first experiment sand was used as a cover substrate, because of a shortage of potting compost. The results are shown in Table 3.26. Although significant differences were found, because of the variability the results are inconclusive.

Table 3.26 Decomposition after exposure to captan in a fruit parcel; p.c. = potting compost; means followed by the same letter in each column do not differ significantly at 5% level according to SNK multiple range test.

treatment	placed before/after treatment	cover substrate	substrate below	decomposition (%) ± s.d.
untreated		p.c.	*soil*	80bc ± 9
20 m west	before	sand	grass	81bc ± 7
10 m west	before	sand	grass	74a ± 14
5 m west	before	sand	grass	78ab ± 12
crop edge west	before	p.c.	soil	89e ± 3
crop, 1.5 kg captan/ha	before	p.c.	*soil*	87de ± 3
crop edge east	after	p.c.	soil	90e ± 4
5 m east	after	p.c.	soil	83cd ± 10
10 m east	after	p.c.	soil	87de ± 4
20 m east	after	p.c.	soil	91e ± 1

In the second experiment the expected treatment did not take place (Table 3.27). No significant differences were found between litterbags. As can be seen, the differences in decomposition between the litterbags are very small.

Table 3.27 Decomposition in an untreated fruit parcel; ANOVA: $P > 0.05$.

treatment	decomposition (%) ± s.d.
untreated	90 ± 3
untreated	89 ± 4
inside parcel	89 ± 5
inside parcel	90 ± 2

In a third experiment with captan, however, an effect could be traced (Fig. 3.36). In this case a correlation was found between distance from the treated parcel and decomposition. In this experiment the captan content of the soil was also estimated. At distances up to 15 m from the treated parcel decomposition was reduced, compared with the untreated control. At the 20 m sampling point decomposition was as high as in the untreated control.

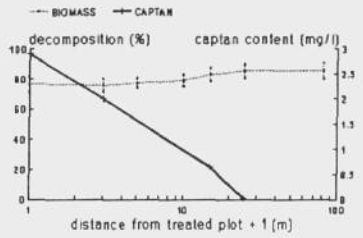


Figure 3.36 Decomposition and captan content at a fruit parcel.

With maneb two experiments were carried out. In the first experiment only treated and untreated litterbags were compared (Table 3.28). The decomposition of the treated litterbags was significantly lower than those untreated. The differences were small, however.

Table 3.28 Decomposition after exposure to maneb in a potato parcel; means followed by same letter in each column do not differ significantly at 5% level according to SNK multiple range test.

treatment	decomposition (%) ± s.d.
untreated	91c ± 3
untreated	88b ± 3
3000 g maneb/ha	85a ± 5
3000 g maneb/ha	86ab ± 4

These results gave cause for a second experiment with litterbags at a range of distances from a potato field. A correlation was found between distance and decomposition (Fig. 3.37). In this case, however, decomposition at the untreated sampling point was much lower than at the sampling point 20 m from the treated plot (Fig. 3.37).

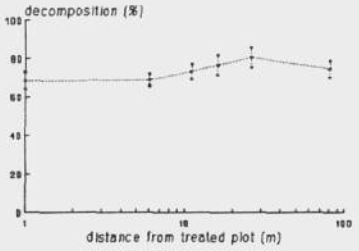


Figure 3.37 Decomposition after maneb treatment in potato.

One experiment was conducted at the Laboratory for Bulb Research on plots disinfected two months before the experiment. In this experiment no effects were detected (Table 3.29). In this case the untreated sampling point was within the parcel, in an area that had not been disinfected.

Table 3.29 Decomposition after exposure to dichloropropene/etridiazole and dichloropropene/metham-sodium in a bulb parcel; ANOVA: $P > 0.05$.

treatment	decomposition (%) ± s.d.
untreated	73 ± 2
untreated	69 ± 4
dichlor./etridiazole	72 ± 3
dichlor./etridiazole	71 ± 5
dichlor./metham-sodium	71 ± 4
dichlor./metham-sodium	71 ± 4

Conclusion
It was concluded that the litterbag method is simple and suitable for tracing the side-effects of pesticides up to 15 m from a treated parcel.

3.4.2.3 Methodic glasshouse experiments

To support the field experiments a number of methodic glasshouse experiments were conducted.

Time-decomposition correlation
First the time-decomposition correlation was established (Fig. 3.38).

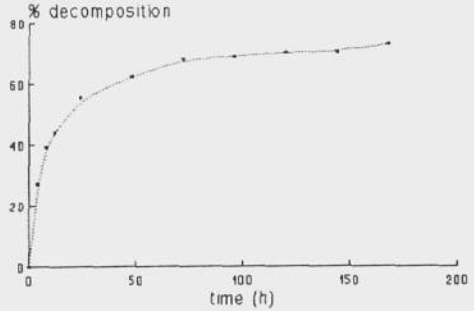


Figure 3.38 Decomposition of cabbage leaf during one week.

The results show that 50% of decomposition takes place within one day. Because dried material was used it was thought that the rapid decomposition during the first day might

be caused by the dissolution of organic matter in soil water. This suspicion was supported with an experiment. A dried leaf disc was placed in sterilized water for four hours; its dry weight decreased by 50%. This effect will probably be smaller in soil, but it may play a part. It is unlikely, however, that the use of pesticides will influence the dissolution of organic matter. The use of fresh material instead of dried leaves would solve the problem. In a preliminary experiment with fresh material decomposition after one week was only 7%; this method is therefore not suitable for tracing the effects of one treatment.

Cover substrate

In another experiment the effects of different substrates to cover the litterbags were studied. The results (Table 3.30) show these differences do not influence decomposition. This result indicate that the fungi responsible for decomposition come from the local soil below. The high decomposition in the uncovered litterbags is probably due partly to the humid weather conditions during this period. In dry periods decomposition is expected to be lower, owing to desiccation.

Table 3.30 Decomposition with different cover substrates; ANOVA: $P > 0.05$.

substrate	decomposition (%) ± s.d.
potting compost	65 ± 5
potting compost	64 ± 4
local soil	65 ± 4
local soil	67 ± 4
uncovered	64 ± 4
uncovered	64 ± 4

Underlying substrate

Normally litterbags were placed on the local soil. For a standard test the use of a more homogeneous soil would be attractive. An experiment was conducted with two centimetres of potting compost on the natural soil. The litterbags were also covered with potting compost.

Table 3.31 Decomposition with different underlying substrates; means followed by same letter in each column do not differ significantly at 5% level according to SNK multiple range test.

substrate	decomposition (%) ± s.d.
potting compost	62a ± 4
potting compost treated with 300 mg/m^2 maneb	61a ± 5
local soil	77b ± 4

The results (Table 3.31) show that decomposition on potting compost is lower than on

natural soil. Furthermore, treatment with the actual field dose of maneb was found to have no effect. For this reason, and in view of the results of the previous experiment, it was decided to continue using local soil as the underlying substrate.

Mesh

The literature shows that larger mesh accelerates decomposition due to invertebrates (Heath *et al.*, 1964). We conducted an experiment with 40-μm gauze and with 1-mm gauze. The results (Table 3.32) show that the litterbags with the larger mesh exhibit less decomposition. No traces of invertebrate activity were found.

Table 3.32 Decomposition with different meshes.

mesh	*decomposition (%) ± s.d.*
40 μm	65b ± 5
40 μm	64b ± 4
1 mm	57a ± 10
1 mm	56a ± 7

3.4.2.4 Validatory glasshouse experiments

CAPTAN

The recommended dose of captan is 150 mg/m^2. From the 1992 field experiments it was found that only 10% of this dose reaches the soil. In the glasshouse experiments, therefore, the recommended dose (150 mg/m^2) and 10% of this dose were tested. Furthermore, an exposure of 5 mg/m^2 was tested, being the lowest dose that showed an effect in the field experiment. The glasshouse experiment was completed by a dose of 1 mg/m^2 and untreated (water only) controls.

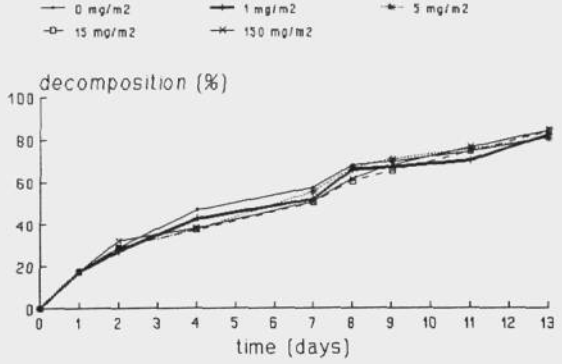


Figure 3.39 *Decomposition in litterbags after exposure with captan at day 1.*

Figure 3.39 shows the results of decomposition at different time intervals post-treatment. The figure shows relatively small differences between treatments. ANOVA showed significant differences between treated and untreated litterbags at three, six and seven days post-treatment. As expected, the 15 and 150 mg/m^2 treatments showed the greatest effects on decomposition. However, in the glasshouse the correlation between dose and effect was not as clear as in the field situation.

Apart from this experiment, the influence of the moment of exposure was investigated. Litterbags were exposed after three and five days to 15 and 150 mg/m^2 captan. After eight days the litterbags were harvested. The results (Table 3.33) show no significant effects. In practice, therefore, it is important to expose the litterbags after one day.

Table 3.33 Decomposition in litterbags after one week; exposure with captan after three and five days; ANOVA: $P > 0.05$.

exposure mg/m^2	decomposition (%) ± s.d.	
	exposure day 3	exposure day 5
0	67 ± 7	
15	66 ± 6	66 ± 7
150	68 ± 6	65 ± 7

Conclusion

It can be concluded that one week after exposure in the glasshouse, effects comparable to the field situation were found. The period in which this effect can be seen, however, is relatively short. If the litterbags are exposed after three or five days no effects are found.

MANEB

In the glasshouse experiments the recommended dose of 300 mg/m^2 was used. Analogous to captan, 10% of the dose was used and concentrations of 10 mg/m^2 and 2 mg/m^2 were applied and compared with a control, treated with water only. Litterbags were exposed after one day.

Table 3.34 Decomposition in litterbags after one week; exposure with maneb after one, three and four days; * = significantly different from untreated ($P < 0.05$).

exposure mg/m^2	decomposition (%) ± s.d.		
	exposure day 1	exposure day 3	exposure day 4
0	77 ± 4		
30	71 ± 4 *	74 ± 3 *	75 ± 4
300	70 ± 4 *	74 ± 3 *	73 ± 4 *

The results are shown in Figure 3.40. ANOVA showed an effect only six days post-treatment. At this time there is a dose-effect relationship, but after the sixth day differences soon disappear. For maneb the effects of exposure after three and, in this case, four days were also investigated. The results are shown in Table 3.34.

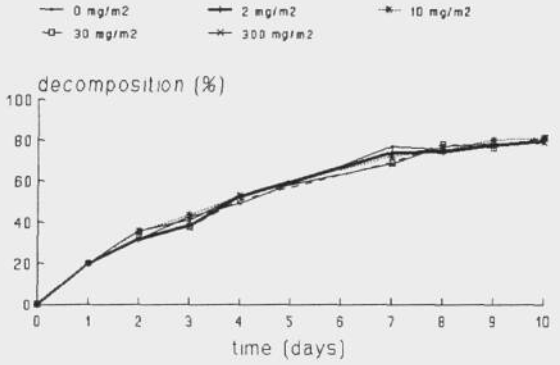


Figure 3.40 Decomposition in litterbags after exposure to maneb at day one.

The results of this experiment show that exposure after three days yielded significant effects as well. After exposure at day four only the highest concentration yielded a significant effect.

Conclusion
From the glasshouse experiments with maneb it can be concluded that, as was found in the field, there is a negative correlation between concentration and decomposition in the litterbags. This effect is only found at one moment (seven days) after the start of the experiment. When the litterbags are exposed after three days, an effect is still found.

3.4.2.5 Methodic field plot experiments 1993 & 1994

As in the glasshouse, in the experimental plot a number of supporting experiments were conducted in order to improve the sensitivity of the tests. The following aspects were studied: cover substrate, decomposition of fresh material and moment of exposure.

Cover substrate
In the glasshouse experiments there appeared to be no influence of cover substrate (uncovered, potting compost and local soil) on decomposition. The use of sand was not tested, however. The use of sand might cause better contact between the litterbag and the pesticide. The results of an experiment with captan (1.5 kg/ha) are shown in Table 3.35.

Table 3.35 Experimental field trial with different cover materials.

cover substrate	treatment	decomposition (%) ± s.d.	difference in decomposition (%) P < 0.001
sand		81 ± 6	8.66
sand	1.5 kg/ha captan	89 ± 3	
potting compost		85 ± 5	7.56
potting compost	1.5 kg/ha captan	92 ± 3	

The results show that, with both sand and potting compost, there is a significant effect of captan treatment. In the case of sand, the difference between treated and untreated is 1% greater than in the case of potting compost. It is therefore recommended to use sand as a cover substrate.

Fresh material as a substrate

The use of fresh material was tested because it is more natural than dried leaves. The test was conducted with cabbage *Brassica oleracea* and lettuce *Lactuca sativa*. The results are shown in Figure 3.41.

The results show a large variation within and between the litterbags, and no correlation with exposure. Therefore, fresh material is not recommended.

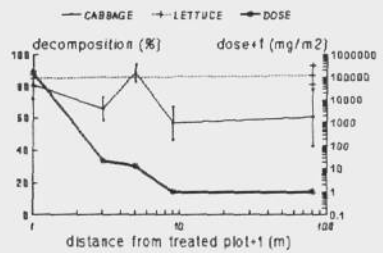


Figure 3.41 Effect of captan treatment on decomposition of fresh material.

Moment of exposure

In the glasshouse the effect of exposure after three or more days was studied; this yielded no better results than after one day. For practical reasons, it would be better to expose the litterbags directly after they are placed in the field. Therefore, an experiment with captan was set up. All litterbags were collected one week after they were placed in the field.

The results are shown in Table 3.36. Only when the litterbags were placed in the field directly after treatment was there no significant difference between treated and untreated bags. The litterbags that were placed in the field directly before treatment showed a

clearer effect than the litterbags placed in the field one day before treatment. Spraying one day before placing the litterbags in the field gives the largest difference. It is recommended to treat the litterbags directly after they are placed. In this case favourable weather conditions can be chosen and there will be certainty that the litterbags are exposed.

Table 3.36 Experimental field trial with different moments of exposure; *** = $P < 0.001$; n.s. = $P > 0.05$.

distance from treated plot (m)	decomposition (%) ± s.d. exposure directly after	directly before	1 day after	1 day before	deposition (%)
0	68 ± 6	73 ± 6	77 ± 6	72 ± 3	100
2	75 ± 4	72 ± 4	84 ± 5		0.01
12		70 ± 9	82 ± 5		0
> 100	78 ± 5	72 ± 5	83 ± 5	86 ± 4	0
difference 0 - >100	9.7 ***	1.2 ns	5.5 ***	14.6 ***	

3.4.2.6 Validatory experiments on experimental plot 1993 & 1994

CAPTAN

In 1993 the litterbags were intended to be exposed one day after being introduced into the field. In the first experiment with captan (22-06-93) the wind direction changed after one day, so that no exposure was expected. We therefore waited until day three for a favourable wind direction. This did not occur, however, and the plot was therefore sprayed with the range of litterbags directed upwind (3 m/s) of the plot. The cover substrate was potting compost.

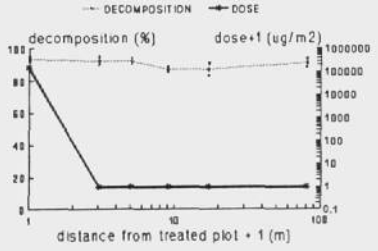


Figure 3.42 Effects of captan treatment 25-06-93 on decomposition; wind speed 3 m/s.

The results (Fig. 3.42) show that, as expected, there is no exposure adjacent to the treated plot. No effect was found; even in the 100%-treated litterbag no inhibition of

decomposition was recorded. This is in accordance with the glasshouse experiment; exposure after three days showed no effect on decomposition. The litterbags at 8 and 16 m from the treated plot showed less decomposition than the other litterbags. This may possibly represent the natural variation in decomposition.

In 1994 the experiment was repeated twice (Figs. 3.43 and 3.44). In the first experiment the spatial variation was studied by using five litterbags with four leaf discs each at each distance, one metre apart. At each distance a litterbag with 20 leaf discs was also used.

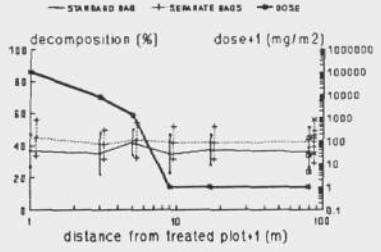


Figure 3.43 Effects of captan treatment 18-04-94 on decomposition; wind speed 3 m/s.

In the first experiment in 1994 the weather was very dry during the week in which the litterbags were in the field. As the results show, decomposition was extremely low. Therefore, these results are inconclusive. When the decomposition in the small separate litterbags are compared with the one large litterbag, no significant differences are found.

In the second experiment in 1994 captan was sprayed after one day. The average decomposition was comparable to most other experiments. There was also a clear correlation between distance and deposition. No correlation was found between the - rather high - deposition of captan and decomposition in the litterbags, however. The results of former experiments were thus not validated.

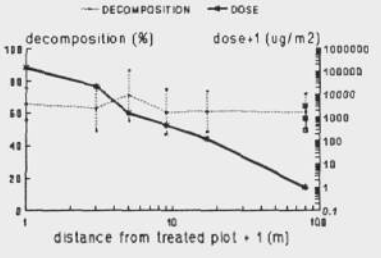


Figure 3.44 Effects of captan treatment 09-06-94 on decomposition; wind speed 5 m/s.

Micro-organisms

In the first experiment the potential effects on fungi were studied as well. Figure 3.45 shows the number of fungi in the litterbags after treatment. No correlation was found between number of fungi and exposure to captan, neither was any correlation found with decomposition of the leaf discs.

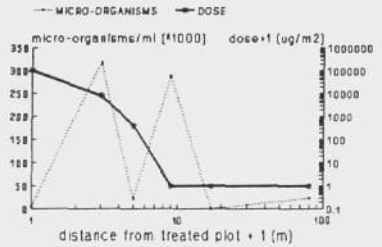


Figure 3.45 Effects of captan treatment 25-06-94 on micro-organisms on cabbage discs; wind speed 3 m/s.

Figure 3.46 shows the results for the micro-organisms in the soil samples at the same sample points as for the litterbags. The results of this experiment also show no clear correlation between exposure to captan and number of micro-organisms.

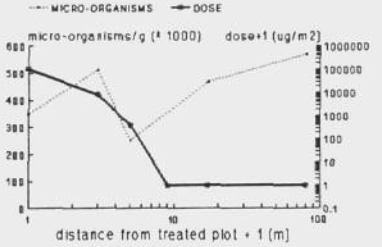


Figure 3.46 Effects of captan treatment 25-06-94 on micro-organisms in soil samples; wind speed 3 m/s.

The last experiment was carried out with petri discs with agar, initialized with a suspension of soil. The results are shown in Figure 3.47. A clear correlation was found between distance from the treated plot and number of fungi. This result at least indicates that the fungicide used is effective against soil fungi.

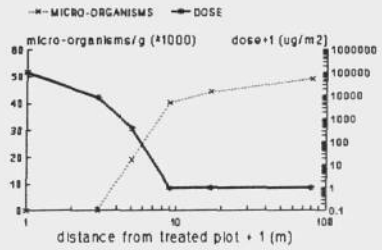


Figure 3.47 Effects of captan treatment 25-06-94 on micro-organisms in agar samples; wind speed 3 m/s.

MANEB

The maneb experiment was conducted under normal conditions; the results are shown in Figure 3.48.

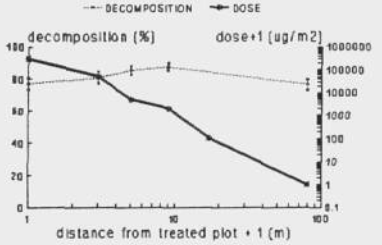


Figure 3.48 Effect of maneb treatment 11-10-93 on decomposition; wind speed 4 m/s.

ANOVA showed significant differences between all distances. The decomposition of the untreated control, however, was as low as the 100%-treated litterbag. It is possible that the soil of the treated control was too different from that in the experimental field, as was the case in the field experiment in 1992. It is therefore hardly possible to draw any conclusion from the experiment with maneb, due to the result of the untreated control.

3.4.3 Discussion and conclusions

Based on the literature, in 1991 and 1992 a method for assessing the side-effects of fungicides on decomposition was developed using litterbags. The first experiments were very promising and correlations were found between exposure to fungicides and decomposition in the litterbags.

In the experimental field, however, these results could not be validated. A number of methodic aspects could be improved. Given the results, a method aimed at decomposition has good potential. The method using litterbags, however, appears to be too sensitive to disturbing factors and does not give readily reproducible results. Therefore, the chances of developing a standard field trial are too slim. One experiment aimed at soil fungi in agar, however, showed a clear correlation with exposure to captan.

Using twenty leaf discs, it appeared to be possible to trace differences of 5% in decomposition between litterbags with 95% confidence. Using the more fundamental approach of the formula, mentioned in Section 3.1, 20-40 leaf discs appear to be necessary to trace differences of 5% from the untreated control. If 10% differences are chosen, 20 leaf discs are sufficient in all cases.

There are several conceivable causes for the failure to validate the results. In the first experiments the differences found were relatively small, so that minor environmental influences will already be sufficient to disturb the experiment. One possible cause can be excluded: the compound was still active, as was apparent from the effects on the fungi in the agar.

A survey of the literature shows that very little is known about the effects of low dosages of fungicides on fungi or on decomposition.

Research carried out in the Netherlands (Arnolds, 1985, 1989; Jansen, 1989) indicates that certain fungi have decreased dramatically in abundance. Although a link with pesticide use has been suggested, this has never been investigated. In a general sense, decline is attributed mainly to habitat loss and acidification. Research on the influence of pesticides is restricted mainly to ectomycorrhizal fungi (ecm's) grown in pure culture. The conclusions are by no means unequivocal; there is considerable variation among the different compounds and species. Copper-based fungicides inhibit the growth of ecm's in pure culture, but not in soil culture. Old country lanes in farming areas often have a far richer macrofungi flora than patches of isolated woodland.

Saprophytic fungi are generally under less threat than mycorrhizal fungi, although species of nutrient-poor soils are threatened by eutrophication. Arboreal fungi and parasites of weakened trees, on the other hand, are increasing in abundance, because of the aging of Dutch forests.

Fokkema (now at IPO) has investigated the influence of fungicides on leaf yeasts, focusing mainly on the effects of fungicides on antagonists (particularly food competitors) of the target moulds (Fokkema and De Nooij, 1981). This study indicates major variation in sensitivity among individual mould species and in the effects of individual compounds. Captafol, captan and the dithiocarbamates were found to have the greatest impact on leaf moulds (Fokkema, 1988). No studies have been performed at levels below practical doses, however.

Bollen, studying soil fungi, reports that at LUW, too, there are no studies focusing on the effects of low concentrations and/or chronic exposure. In a review article (Bollen, 1979) he reports extremely rapid recovery of soil microflora via i) more tolerant species

replacing more sensitive species, ii) rapid recolonization, and iii) formation of resistant strains. This study indicates that effects on processes such as those measured by means of litterbags will be difficult to measure.

Trappe et al. (1984) have published a review article on the impact of pesticides on mycorrhizal fungi. Their major conclusion is that existing studies are often poorly comparable and ambiguous in their conclusions. At low concentrations soil fumigants appear to have little impact, while at higher concentrations some decline is generally found.

Unestam et al. (1989) report that effects found in the laboratory with ectomycorrhizal fungi cannot be translated to the field situation, one reason being that in the laboratory the root itself is affected by the fungicide being tested.

From our studies and from literature, it can be concluded that side-effects of pesticide drift on fungi are to be expected. If effects on the decomposition process occur in the field, however, is to be doubted.

4 GUIDELINES FOR BIOASSAYS

4.1 Introduction

In this chapter guidelines are given for duckweed (Section 4.2), *Chaoborus* and *Gammarus* (Section 4.3), *Brassica napus* and *Poa annua* (Section 4.4) and *Pieris brassicae* (Section 4.5). The guidelines are based partly on the results of our studies and related literature, but also on some general principles elaborated in a previous study (De Jong *et al.*, 1990).

General requirements

The guidelines must meet a number of general requirements which, although seemingly trivial, will be stated explicitly for the sake of completeness. In the first place the trials should yield unambiguous results, i.e. it should be clear whether an observed effect is to be attributed to pesticide use. This has implications for experimental design: it necessitates use of a control (untreated or treated), for example. The design (number of organisms, number of replicates) should also allow for observation of (differences in) effects with an acceptable degree of reliability. Furthermore, the influence of other factors or combined effects should be excluded as far as possible. The field trials should also allow conclusions to be drawn about the practical field situations in which the pesticide is to be (or may be) applied. This means that the crop to be protected must be grown at the test site and that the dosage and method of application must be similar to those used under practical circumstances.

Specific premises
In their basic structure, the proposed field trials follow the guidelines in force for efficacy testing (PD, undated). Our guidelines are thus built up as follows: 1. Experimental conditions, 2. Application of compounds, and 3. Observation. In line with the NEN standards, an additional section has been added: 4. Validity.

Experimental conditions

Reference
In all cases, the exposed bioassays should be compared with an unexposed bioassay. This reference must have undergone the same mechanical operations, weather conditions, etc.

Treated control
In addition to an untreated reference, a treated control is also desirable. This is a bioassay treated with a 100% dose of the pesticide under study. This enables it to be established whether or not there are unusual conditions leading to the absence of effects.

Trial duration
The duration of the trial depends on the anticipated effects. To draw maximum benefit from the potential offered by a field trial, however, it is desirable to continue a field trial for at least one (field) season, or one generation of the test organism. This also means

that the pesticide can be applied several times, in accordance with practical use. In addition, any medium- to long-term effects can also thus be traced.

The minimum duration is the period after which effects become visible in the treated control. An experiment can be ended when no new effects are found in the exposed units and may be of longer duration if recovery is part of the research question.

Pesticide application

Dosage
In all trials, it is proposed to apply the highest recommended dose, for this will, in principle, constitute the greatest hazard occurring in normal use. In practice, however, there are several circumstances that may lead to (locally) higher loads, for instance when spraying zones overlap. For this reason, it is also proposed to treat a trial plot with two times the maximum dose prescribed, thus incorporating a worst-case situation into the field trial. This also allows for use - deliberate or not - of a dose in excess of the highest prescribed dose. At the same time, the chances of effects not being observed because of unforeseen circumstances are thus reduced. A problem may arise if the maximum dose produces no observable effect, while the twofold dose does. In this case, there is evidently a potential hazard, which is not apparently encountered during normal use. In this case, a solution may perhaps be found in higher safety margins, to be achieved by prescribing lower user doses, for instance. A study can also be conducted using graded doses of the pesticide under review, enabling a dose-effect relationship to be established. For this type of study, a different, more comprehensive test method is required, however. A field trial employed in the framework of pesticide approval should lead to a firm conclusion on the occurrence or non-occurrence of side-effects. For this reason, the maximum dose and two times this dose are used in the standard trials. Once a hazard is found to exist, more accurate tests can always be carried out if so desired.

Formulation and method of application
The use of a certain formulation or method of application can be hazardous. The same formulation and/or method of application should therefore be employed in the field trial. If other formulations and/or methods of application likewise involve a hazard, the field trial should initially focus on the situation in which the greatest hazard is anticipated. If necessary, a separate trial should be performed for the other situations. As regards exposure of organisms outside the target area, attention should be focused on those situations in which exposure will be highest.

In addition, there are some applications in which the load on ditches and field margins may be as high as 100%, for instance when ditch banks and ditches themselves are treated. In such cases, however, the ditches and ditch banks constitute the target area, and the effects can no longer be termed side-effects. The desirability of such treatment should be debated in another context. A loading of 100% may also occur if aerial spraying is employed. In this case, there will often already be a serious hazard, obviating the need for a field trial. If a moderate hazard is anticipated, a field trial can be conducted; for the time being, however, we have opted for field trials focusing on more standard pesticide use.

Observation

The methods of observation depend primarily on the effect anticipated. In general, mortality will be the first parameter to assess. However, growth, pupation, moulting etc. may also be assessed, depending on the organism and the effect anticipated. Effects should be monitored from the moment they become measurable in the treated control.

In a field trial, it is always essential to know whether the experimental organisms are actually exposed. Although a treated control yields important information on this point, measurement of pesticide concentrations or depositions in the exposed environmental compartments and in the organisms tested should be part of the standard procedure, as these data can provide support in establishing causal relationships.

Validity

In the United States, the EPA has drawn up Standard Evaluation Procedures (SEP) for test results. In principle, there is a separate SEP for each test. These procedures ask detailed questions about the way the test is carried out. If a detailed guideline is available, this means that the SEP strongly resembles this guideline, the difference being that it is in the interrogative form. In view of the level of detail of the guidelines proposed here, there would appear to be less need for such an evaluation. In our opinion, a number of points can be distinguished that are of importance in evaluating test results. These can be summarized as follows:

Statistical significance
Effects must be demonstrated with 95% (two-sided) certainty. This condition places high demands on the test method. In a number of cases, it will be necessary to determine the variation in the bioassay in the field prior to the trial. Using these results, the number of random tests required for obtaining a statistically verifiable result can be determined. In this context, it is also important what differences are to be demonstrated. The premise here is that it should be possible to establish 10% differences from the untreated control.

Effects in the untreated control
In laboratory tests, mortality in the untreated control may not exceed 10%. In the field, this figure will depend on the natural mortality rate. One way to assess the natural mortality rate is to transfer the laboratory organisms to an untreated field. The mortality rate of these organisms can then serve as a standard for untreated mortality in the field. In several places in the literature, a field mortality rate of 15% in the untreated control is quoted as being acceptable (e.g. EPPO, 1992).

Effects in the treated control
In the treated control, a 80-100% effect should be found, in conformity with the laboratory tests. This effect may be mortality or another anticipated effect.

In field testing, a conflict may arise between practicability and compliance with the basic premises. A test yielding a conclusion within the proposed statistical margins may prove to be too comprehensive (= too costly), but a test of limited scope may lead to greater

margins of uncertainty. To solve this dilemma, a trial can be focussed on a worst-case situation. By applying a higher dose, the scope of the test can be limited. If effects are not then demonstrated, it may be assumed that the practical dose will not give rise to effects, either. If effects are found, however, there will have to be very careful translation to the practical dose.

The guideline proposals aim, provisionally, at testing the effects of a single pesticide on a single (group of) organism(s). On each of these aspects, the scope of the guidelines could be extended. In principle, the same trials could also be used for evaluating the effects of a combination of pesticides. There may be various reasons for combined testing. In an existing practical application involving multiple treatment, an old pesticide may be replaced by a new compound. Alternatively, there may be suspicions of combined toxicity or synergistic effects. In such cases, the pesticides involved can be tested simultaneously.

Effects on more than one group of organisms can be assessed by combining different tests or elements of tests.

4.2 Proposed guideline for a field bioassay for evaluating the side-effects of pesticides on duckweeds *Lemna minor, lemna gibba* and *Spirodela polyrhiza*

4.2.1 Experimental conditions

Test species

Trials should be performed with the duckweed species *Lemna minor*, *Lemna gibba* and *Spirodela polyrhiza*. Tests are conducted preferably with fronds from laboratory cultures, grown at 20°C and with a 16-hr photoperiod. If not available, fronds may be obtained from exponentially growing populations in the field near to the experimental plot.

Field site

Tests are performed in ditches surrounding an agricultural plot with a growing crop requiring the treatment under investigation. No pesticide spraying is allowed within 500 m of the plot. The ditches in which the tests are performed may not drain areas with pesticide use.

Bioassay units

Growth tests with duckweed are performed using floating bioassay units. The following construction is proposed. A floating unit consists of 5x5 or 3x8 mini-compartments of transparent PVC sheet, each of which is 15x15 cm wide and 6 cm high. The tops and bottoms of the compartments are open. The compartments are fixed on a frame of 18-mm PVC pipe glued with prefab bends. The unit must be placed in the ditch inside a cage (made of nylon bird netting, for example) to exclude birds and frogs. For each duckweed species, eight compartments are filled with fronds at a 10% cover rate.

Experimental lay-out

A schematic view of the field lay-out is given in Figure 4.1. Floating bioassay units are placed at each side of the field to be sprayed. Two unexposed control units are placed at 100 m distance in ditches that are not draining the field to be sprayed. A treated control unit is placed in an isolated section of a ditch (enclosure) that is treated with a plant sprayer at the field rate. The enclosure consists of a cylinder with a removable polyethylene layer on the inner side, and is driven firmly into the ditch bed. Various construction materials are allowed.

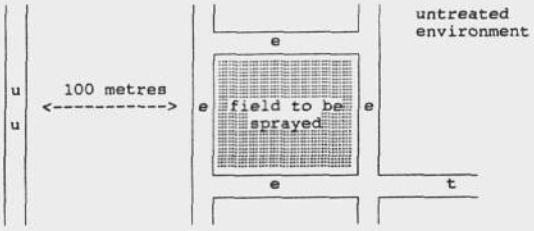


Figure 4.1 Schematic view of the field lay-out. Explanation of codes: e=exposed floating unit; u=unexposed floating unit (untreated control); t=treated floating unit inside an enclosure (treated control).

Tests should be performed in duplo. To exclude the possibility of mutual spraying impact, the test fields should be located at least 500 metres apart.

Sample size
The number of floating compartments per sample point needed to demonstrate a desired difference between treatments can be determined using the formula (Sokal & Rohlf, 1981):

$$n \geq 2(\sigma/\delta)^2 * \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$$

where
n = number of replications
σ = true standard deviation
δ = smallest true difference to be detected
v = degrees of freedom
α = significance level
P = desired probability that a difference will be found to be significant
$t_{\alpha[v]}$ and $t_{2(1-P)[v]}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively.

If results are statistically tested at the 95% confidence level, then $u=1.96$.

4.2.2 Application

Compounds
Apply the compounds being studied according to the manufacturer's guidelines, in a formulation used in everyday practice. If a worst case scenario is needed a dosage of two times the recommended dose can be used.

Equipment
Apply the compounds using normal equipment, according to standard practice.

Spraying schedule
Carry out spraying at a time at which the compound is normally applied. If possible, spray the various duplos simultaneously, but at least within a two-hour period, in order to avoid differences in weather conditions.

Meteorological conditions
Field trials should be conducted in dry weather and with a moderate wind towards the bioassay units. Extremes, such as prolonged drought or rain, should be avoided.

4.2.3 Observations

Fate
Droplet deposition is measured in triplo, using 12.5 x 2.5 cm water-sensitive paper strips

at each sample point. Pesticide residue levels must also be measured in the water column at a standard depth at 1 hr pre-treatment, and at 1 hr and 1, 2, 4, 7, 14, 21 and 28 days post-treatment.

Meteorological conditions
Record the meteorological conditions at the time of spraying: precipitation, temperature, wind, cloud cover, sunshine and air humidity (incl. leaf moisture) at the experimental site.

Water movement
Water movement must be measured continuously.

Nutrients
Nitrate and ortho-phosphate must be measured pre-treatment and weekly post-treatment. Each water sample should be taken as a mixture of three subsamples.

Bioassay responses
Growth response, recorded as the difference in cover rate between two time-points, is measured weekly until 28 days post-treatment. Cover rate is estimated visually to the nearest 5% by pushing the fronds to one side of the compartment, using a 15 cm wide PVC sheet. Growth rate is calculated according to the equation:

$$g = \frac{lnC_{t_2} - lnC_{t_1}}{t_2 - t_1}$$

in which:

g = growth rate (fraction per day)
t = day number
C = duckweed cover (%)

If the cover rate exceeds 50%, it must be reset to 10%. Growth rate must then be corrected for the fraction by which the cover rate is reset. Other types of damage to the fronds must also be recorded, such as necrotic and chlorotic damage, and malformation of the roots. The types of damage to be investigated can be based on expectations regarding specific effects resulting from the mode of action of the compound under investigation.

4.2.4 Statistical test procedures

ANOVA
Considering the test ditches as treatments, an ANOVA (analysis of variance) test is performed on growth rates with nutrients as covariates, followed by the Student-Newman-Keuls procedure to compare among means. Homogeneity of variances is checked with Bartlett's test.

Regression analysis

For each time interval, a regression analysis is performed on the growth rates versus deposition rates, using the log-logistic model:

$$E = \frac{E_{max}}{1 + \alpha . e^{-\lambda D}}$$

in which:

E = effect observed (% growth inhibition)
E_{max} = effect maximum (100%)
α, λ = parameters to be estimated
D = ln-transformed deposition rate (ln{% of field rate})

From this model ED_{50} for growth inhibition can be calculated.

Validity

Test results are not valid if results are unclear, or if there is high observable impact in the untreated controls or little impact in the treated controls. Results are also invalid if bioassay units already showed significant differences in the measured parameters prior to treatment. Results are not valid if no deposition is found at sample points.

4.2.5 Cost

	net working days	material expenses
1 materials	10	
- test organisms		500
- test plots		500
- field equipment		( )
2 sampling		
- test organisms	n.a.	
- abiotic parameters	5	
3 parameter measurement		
- test organisms	5	500
- deposition measurements	2	500
- pesticide residue analysis	3	500
4 data interpretation	5	( )
5 completion of test form	5	n.a.
total working days	about 35	n.a.
cost estimate (subtotal)	Dfl. 35,000	Dfl. 2,500
overall cost (very rough estimate):	Dfl. 37,500	

( ) depreciation cost of materials still to be included
n.a. not applicable.

4.3 Proposed guideline for a field bioassay for evaluating the side-effects of pesticides on midge larvae *Chaoborus crystallinus* and amphipods *Gammarus* spp.

4.3.1 Experimental conditions

Test species

Since laboratory culture of *Chaoborus crystallinus* is difficult, third- and fourth-instar larvae may be obtained from fishless ponds or from ditches that have no history of pesticide exposure. The species composition of the sampled population should be checked for by determination of at least twenty larvae. Prior to a trial, the larvae should be fed *ad libitum* on daphniids and copepods.

For *Gammarus* it is suggested to perform trials with a common species, such as *G. pulex* or *G. tigrinis*. Animals may be obtained from the laboratory, cultured at 20°C and with a 16-hr photoperiod. Alternatively, animals may be obtained from a ditch that has no history of pesticide exposure near the field site. Juveniles of 2-3 mm are preferable to larger sizes. Prior to a trial, the animals should be fed *ad libitum* on decaying chestnut leaves.

Field site

Tests are performed in ditches surrounding an agricultural plot with a growing crop that requires the treatment under investigation. No pesticide spraying is allowed within 500 m of the plot. The ditches in which the tests are performed may not drain areas with pesticide use.

Bioassay units

Test animals are held in exposure chambers, consisting of a 350-ml glass jar screwed to a bag of 280-μm stainless steel mesh (Fig. 4.2). Air enclosed in the glass provides buoyancy to the chamber and forms an oxygen buffer for the test animals. Ten animals are tested in one chamber. *Gammarus* is fed on processed chestnut leaves during the trial.

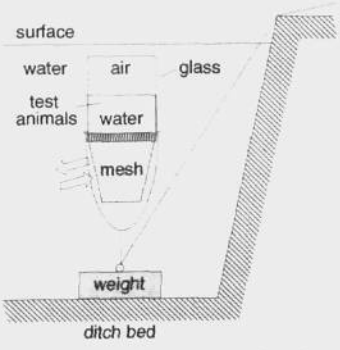


Figure 4.2 Schematic view of the exposure chamber.

If feeding response is measured (Section 2.2.5), the food is offered as a standard-sized leaf disc of known fresh weight. A leaf disc is then replaced by a new disc at each observation time, in order to determine the feeding rate at the different observation intervals. *Chaoborus* is not fed during the trial.

Experimental lay-out

A schematic view of the field lay-out is given in Figure 4.3. Floating bioassay units are placed on each side of the field to be sprayed. Two unexposed control units are placed at 100 m distance in ditches that are not draining the field to be sprayed. A treated control unit is placed in an isolated section of a ditch (enclosure) that is treated with a plant sprayer at the field rate. The enclosure consists of a cylinder with a removable polyethylene layer on the inner side, and is driven firmly into the ditch bed. Various construction materials are allowed.

Tests should be performed in duplo. To exclude the possibility of mutual spraying impact, the test fields should be located at least 500 metres apart.

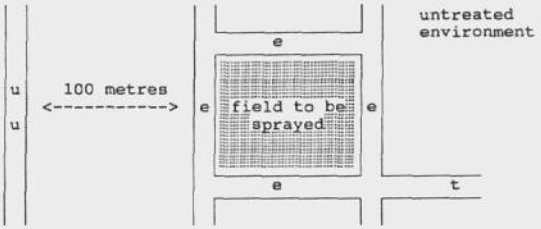


Figure 4.3 Schematic view of the field lay-out. Explanation of codes: e=exposed set of chambers; u=unexposed set of chambers (untreated control); t=treated set of chambers inside an enclosure (treated control).

Sample size

The number of exposure chambers per sample point needed to demonstrate a desired difference between treatments can be calculated using the formula (Sokal & Rohlf, 1981):

$$n \geq 2(\sigma/\delta)^2 * \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$$

where

n = number of replications

σ = true standard deviation

δ = smallest true difference to be detected

v = degrees of freedom

α = significance level

P = desired probability that a difference will be found to be significant

$t_{\alpha[v]}$ and $t_{2(1-P)[v]}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively.

If results are statistically tested at the 95%-confidence level, then $u=1.96$.

4.3.2 Application

Compounds
Apply the compounds being studied according to the manufacturer's guidelines, in a formulation used in everyday practice. If a worst-case scenario is needed, a dosage of two times the recommended dose can be used.

Equipment
Apply the compounds using normal equipment, according to standard practice.

Spraying schedule
Carry out spraying at a time at which the compound is normally applied. If possible, spray the various duplos simultaneously, but at least within a two-hour period, in order to avoid differences in weather conditions.

Meteorological conditions
Field trials should be conduced in dry weather and with a moderate wind towards the bioassay units. Extremes, such as prolonged drought or rain, should be avoided.

4.3.3 Observations

Fate
Droplet deposition is measured in triplo, using 12.5 x 2.5 cm water-sensitive paper strips at each sample point. Pesticide residue levels must also be measured in the water column at a standard depth at 1 hr pre-treatment, and at 1, 3, 6, 12, 24, 48, 96 and 168 hr post-treatment.

Meteorological conditions
Record the meteorological conditions at the time of spraying: precipitation, temperature, wind, cloud cover, sunshine and air humidity (incl. leaf moisture) at the experimental site.

Water movement
Water movement must be measured continuously.

Dissolved oxygen, pH, and EC
At each site, continuous measurements of dissolved oxygen levels, pH, and EC are recommended. If this is not possible, these variables must be measured twice daily, in the early morning and in the afternoon.

Bioassay responses
Responses of the test animals are recorded at 24, 48, 96 and 168 hr post-treatment. For both species, the effects to be recorded are mortality and immobilization. For *Gammarus*, it is suggested also to measure the feeding response. Food consumption is then determined as the difference in fresh weight of the food (a standard-sized chestnut leaf disc) at each observation interval.

4.3.4 Statistical test procedures

ANOVA

Considering test ditches as treatments, ANOVA (analysis of variance) tests are performed on mortality and immobilization percentages, and on feeding rates, followed by Student-Newman-Keuls multiple means comparison procedures to compare among means. For mortality and immobilization, tests are performed on arcsine square root transformed data. Homogeneity of variances is checked with Bartlett's test.

Regression analysis

Regression analyses are performed on the deposition rates versus the cumulative mortality and immobilization percentages at 168 hr, using the log-logistic model:

$$E = \frac{E_{max}}{1 + \alpha . e^{-\lambda . D}}$$

in which:

E = effect observed (% mortality or immobilization)
E_{max} = effect maximum (100%)
α, λ = parameters to be estimated
D = ln-transformed deposition rate (ln{% of field rate})

From this model LD_{50} and ED_{50} for immobilization can be calculated.

Validity

Test results are not valid if results are unclear, or if there is high observable impact in the untreated controls or little impact in the treated controls. Results are also invalid if bioassay units already showed significant differences in the measured parameters prior to treatment. Results are not valid if no deposition is found at sample points.

4.3.5 Cost

	net working days	material expenses
1 materials	10	
- test organisms		500
- test plots		500
- field equipment		( )
2 sampling		
- test organisms	n.a.	
- abiotic parameters	5	
3 parameter measurement		
- test organisms	5	500
- deposition measurements	2	500
- pesticide residue analysis	3	500
4 data interpretation	5	( )
5 completion of test form	5	n.a.
total working days	about 35	n.a.
cost estimate (subtotal)	Dfl. 35,000	Dfl. 2,500
overall cost (very rough estimate):	Dfl. 37,500	

( ) depreciation cost of materials still to be included
n.a. not applicable.

4.4 Proposed guideline for a field bioassay for evaluating the side-effects of pesticides on terrestrial vascular plants *Brassica napus* and *Poa annua*

4.4.1 Experimental conditions

Test species
Trials should be performed with at least the dicotyledonous species *Brassica napus* and the monocotyledonous species *Poa annua*. Test should be performed using laboratory (glasshouse, climate chamber) reared plants obtained from homozygotic plant seeds. Tests should be performed with plants 1-2 weeks old, with in the case of *B. napus* at least two leaves appearing apart from the dicotyledons, and in the case of *P. annua* the blades being at least 5 cm high.

Field site
Experiments are conducted using a field plot of at least 100x100 m. At this field site a crop should be grown requiring the treatment under investigation.

Bioassay units
For *B. napus* 50x33 cm trays with 150 compartments of 2.5 cm ø can be used. This tray is put in a 60x40 cm transport-container, filled with hydro-grains and a hydro-mat, to ensure a good water supply. For *P. annua*, 10-l flower boxes can be used. The water supply is ensured by a second box below the unit connected with the hydro-mat by means of slots (Fig. 4.4). In the box three areas are sown with 0.075 g of grass-seed. Any type of well-mixed potting compost can be used as a substrate, provided it is uncontaminated with pesticide residues. If necessary, units can be covered with 1-cm wire after spraying to prevent predation by birds or mammals. Bioassays can be brought to the field on the day of spraying and brought back to the laboratory to study effects at least two hours after application.

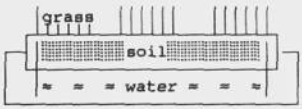


Figure 4.4 Bioassay unit with *Poa annua*.

Experimental lay-out
Two bioassay units are placed inside the treated plot, at 2, 4, 8 and 16 m downwind of the plot, and an unexposed control is placed at least 500 m from the treated plot (see Fig. 4.5).

Tests should be performed in duplo. To exclude the possibility of mutual spraying impact, the test fields should be located at least 500 metres apart.

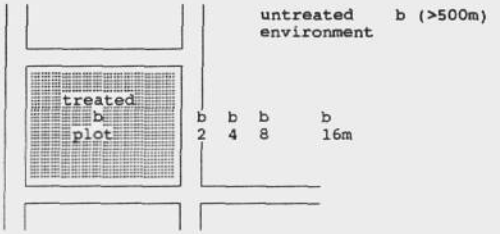


Figure 4.5 Field lay-out for terrestrial studies (b = bioassay unit).

Sample size
The number of replications needed to demonstrate a desired difference between treatments can be determined using the formula (Sokal & Rohlf, 1981):

$$n \geq 2(\sigma/\delta)^2 * \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$$

where
n = number of replications
σ = true standard deviation
δ = the smallest true difference that is desired to detect
v = degrees of freedom
α = significance level
P = desired probability that a difference will be found to be significant
$t_{\alpha[v]}$ and $t_{2(1-P)[v]}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively.

If results are statistically tested at the 95%-confidence level, then $u=1.96$.

In several experiments, differences of 10% could be traced using twenty plants.

4.4.2 Application

Compounds
Apply the compounds being studied according to the manufacturer's guidelines, in a formulation used in everyday practice. If a worst-case scenario is needed, a dosage of two times the recommended dose can be used.

Equipment
Apply the compounds using normal equipment, according to standard practice.

Spraying schedule
Carry out spraying at a time at which the compound is normally applied. If possible, spray the various plots simultaneously, but at least within a two-hour period, in order to avoid differences in weather conditions.

Meteorological conditions
Field trials should be conduced in dry weather and with a moderate wind towards the bioassay units. Extremes such as prolonged drought or rain should be avoided.

4.4.3 Observations

Fate
Droplet deposition is measured in triplo, using 12.5 x 2.5 cm water-sensitive paper strips at each sample point.

Meteorological conditions
The meteorological conditions are recorded at the time of spraying: precipitation, temperature, wind, cloud cover, sunshine and air humidity (incl. leaf moisture) at the experimental site.

Bioassay responses
One-third of the plants is harvested at the moment a clear visible effect is observed on the 100%-treated unit. One week after this, the next one-third is harvested, followed after two weeks by the last plants. For each plant the fresh and dry weight should be determined and phytotoxic symptoms such as chlorosis, necrosis, wilting, leaf or stem damage, anomalous growth or development and any other observable damage should be recorded.

4.4.4 Statistical test procedures

ANOVA
Considering the distances from the experimental plot as treatments, an ANOVA (analysis of variance) test can be performed on biomass, followed by the Student-Newman-Keuls procedure to compare between means. Homogeneity of variances is checked with Bartlett's test.

Regression analysis
For each time interval, a regression analysis can be performed on the growth rates versus deposition rates and distance. The following model can be applied for calculating plant weight (W) (Streibig, 1988):

$$W = (W_{untr}-W_{max})/(1+EXP(-2(a+b\,LOG(dist))))+W_{max}$$

in which W_{max} is the plant weight at the 100%-treated control
W_{untr} is the response of the untreated control
a and b are constants
dist is the distance from the treated plot

From this model the $Edist_{50}$ (Edist = effect distance) value can be calculated. At the 50% effect distance the corresponding deposition rate can be calculated from the deposition measurements. The deposition can estimated from the three or four sample points nearest to the $Edist_{50}$ according to the model:

Deposition = EXP(*c*-*d*LOG(dist))

in which *c* and *d* are constants
dist = *distance*

The results of this calculations are the ED_{50} for distance and for deposition.

Validity

Test results are not valid if results are unclear, or if there is high observable impact in the untreated controls or little impact in the treated controls. Results are also invalid if bioassay units already showed significant differences in the measured parameters prior to treatment. Results are not valid if no deposition is found at sample points.

4.4.5 Cost

	net working days	material expenses
1 materials	5	
- test organisms		500
- test plots		1,000
- field equipment		( )
2 sampling		
- test organisms	1	
- abiotic parameters	1	
3 parameter measurement		
- test organisms	12	500
- deposition measurements	2	500
4 data interpretation	5	( )
5 completion of test form	5	n.a.
total working days	about 31	n.a.
cost estimate (subtotal)	Dfl. 31,000	Dfl. 2,500
overall cost (very rough estimate):		Dfl. 33,500

( ) depreciation cost of materials still to be included
n.a. not applicable.

4.5 Proposed guideline for a field bioassay for evaluating the side-effects of pesticides on *Pieris brassicae* caterpillars

4.5.1 Experimental conditions

Test species
Trials should be performed with caterpillars of *Pieris brassicae*. Tests should be performed using laboratory (glasshouse, climate chamber) reared organisms at 21°C. Test organisms can be reared on any appropriate *Brassica* species, but the same species should be used as a host plant during and after exposure. Tests should be performed using first instar larvae (48-72 hours of age).

Field site
Experiments are conducted using a field plot of at least 100x100 m. At this field site a crop should be grown requiring the treatment under investigation.

Bioassay units
Larvae, 48-72 hours old, are placed on whole *Brassica* plants and exposed to the test compound. After exposure the plants are covered with 0.5-mm gauze. Plants should be large enough to follow the effects on caterpillars until pupation. Bioassays can be brought to the field on the day of spraying and brought back to the laboratory to study effects at least two hours after application.

At each sample point at least ten plants with ten caterpillars each are used.

Experimental lay-out
Two bioassay units are placed inside the treated plot, at 2, 4, 8 and 16 m downwind of the plot, and an unexposed control is placed at least 500 m from the treated plot (see Fig. 4.6).

The test should be performed in duplo. To exclude the possibility of mutual spraying impact, the test fields should be located at least 500 metres apart.

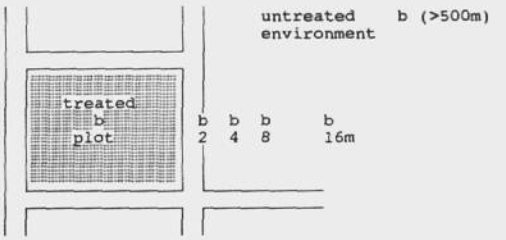


Figure 4.6 Field lay-out for terrestrial studies (b = bioassay unit).

Sample size
The number of replications needed to demonstrate a desired difference between treatments can be determined using the formula (Sokal & Rohlf, 1981):

$$n \geq 2(\sigma/\delta)^2 * \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$$

where
n = number of replications
σ = true standard deviation
δ = the smallest true difference that is desired to detect
v = degrees of freedom
α = significance level
P = desired probability that a difference will be found to be significant
$t_{\alpha[v]}$ and $t_{2(1-P)[v]}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively.

If results are statistically tested at the 95%-confidence level, then $u=1.96$.

4.5.2 Application

Compounds
Apply the compounds being studied according to the manufacturer's guidelines, in a formulation used in everyday practice. If a worst-case scenario is needed a dosage of two times the recommended dose can be used.

Equipment
Apply the compounds using normal equipment, according to standard practice.

Spraying schedule
Carry out spraying at a time at which the compound is normally applied. If possible, spray the various duplos simultaneously, but at least within a two-hour period, in order to avoid differences in weather conditions.

Meteorological conditions
Field trials should be conduced in dry weather with a moderate wind towards the bioassay units. Extremes such as prolonged drought or rain should be avoided.

4.5.3 Observations

Fate
Droplet deposition is measured in triplo, using 12.5 x 2.5 cm water-sensitive paper strips at each sample point.

Meteorological conditions
The meteorological conditions are recorded at the time of spraying: precipitation, temperature, wind, cloud cover, sunshine and air humidity (incl. leaf moisture) at the experimental site.

Bioassay responses
Caterpillar survival is recorded daily until pupation. The number of butterflies emerging is also recorded, as are any visible effects other than mortality.

4.5.4 Statistical test procedures

ANOVA
Considering the distances from the experimental plot as treatments, a Kruskall-Wallis analysis of variance test can be performed on survival, followed a pairwise test (Mann-Whitney-U) to determine which exposure rates differed significantly (Siegel, 1988).

Regression analysis
LD_{50} values can be estimated using the following logistic-model, calculating the mortality percentage (M):

$$M = (M_{max}\text{-}M_{untr})/(1+ae^{-b\mathrm{LOG}dist}) + M_{untr}$$

in which M_{max} is the maximal response (100% mortality)
M_{untr} is the mortality in the untreated control units
a and b are constants
dist is distance

From this model the $Ldist_{50}$ (Ldist = lethal distance) value can be calculated. At the 50% lethal distance the corresponding deposition rate can be calculated from the deposition measurements. The deposition can estimated from the three or four sample points nearest to the $Ldist_{50}$ according to the model:

$$\text{Deposition} = \mathrm{EXP}(c\text{-}d\mathrm{LOG}(\text{dist}))$$

in which *c* and *d* are constants
dist = distance

The results of this calculations are the LD_{50} for distance and for deposition.

Validity
Test results are not valid if results are unclear, or if there is high observable impact in the untreated controls or little impact in the treated controls. Results are also invalid if bioassay units already showed significant differences in the measured parameters prior to treatment. Results are not valid if no deposition at sample points is found.

4.5.5 Cost

	net working days	material expenses
1 materials	10	
- test organisms		500
- test plots		1,000
- field equipment		( )
2 sampling		
- test organisms	1	
- abiotic parameters	1	
3 parameter measurement		
- test organisms	10	500
- deposition measurements	2	500
4 data interpretation	5	( )
5 completion of test form	5	n.a.
total working days	about 34	n.a.
cost estimate (subtotal)	Dfl. 34,000	Dfl. 2,500
overall cost (very rough estimate):		Dfl. 36,500

( ) depreciation cost of materials still to be included
n.a. not applicable.

5 DISCUSSION AND CONCLUSIONS

5.1 Discussion

In Chapters 2 and 3 the results of each bioassay were discussed. In this chapter the bioassays are discussed at a more general level, encompassing the various possible functions of bioassays, interpretation of bioassay results for free-living populations and at higher levels of ecological organization, short- and long-term effects, and recovery from effects. The scope for implementing bioassays in pre- and post-registration procedures for pesticides will be discussed in a future report.

Purposes of bioassays

Field bioassays can be used for various purposes. They may be used to register industrial discharges (Van de Guchte, 1988), or for educational purposes aimed at changing farmers' spraying behaviour. In these applications field bioassays are used as a warning system to indicate that 'something' is wrong. Consequently, the effects do not necessarily need to be representative for the effects occurring in the field. When the method is applied in risk analysis, which aims at ecosystem protection, demands are higher. In this application questions arise as to whether or not the effects in the bioassay are representative for the effects on free-living populations of the same species and taxonomically related species; in addition, effects on functional relationships between species, so-called indirect effects (De Snoo *et al.*, 1994), become relevant.

Bioassays can also be used for determining the effectiveness of drift-reducing measures or for determining a safe buffer zone width (Marrs *et al.*, 1992; Davis *et al.* 1993).

More generally, bioassays sometimes appear to be more sensitive than deposition measurements. For this reason bioassays also constitute a sensitive method for detecting pesticide deposition.

Extrapolation of bioassay results

In the aquatic environment, in the case of *Chaoborus* the literature gives some indication that effects in a field bioassay do represent effects on field populations. Helgen *et al.* (1988) found that spraying of the insecticide temephos into a natural pond gave approximately the same decrease in the free-living *C. americanus* population as the mortality rate seen in the field bioassay. With respect to the sensitivity of related species, a report by Eisler (1992) is relevant. Treatments of lakes with diflubenzuron at rates between 2.5 and 16 µg/l effectively control Diptera, including Chaoboridae, Chironomidae and Culicidae. Therefore, *C. crystallinus* may have some value as an indicator for the effects of diflubenzuron on species within these three families. The indicator function of *C. crystallinus* using methyl-parathion may be less pronounced, as this compound has a less selective mode of action.

The possible role played by *Chaoborus* in the occurrence of indirect effects is linked to its role in aquatic communities. Arnott & Vanni (1993) showed that *Chaoborus* primarily influences the zooplankton community structure, overruling influences due to competition among zooplankton species. Consequently, if *Chaoborus* populations are affected by an

insecticide, major shifts within the community structure may occur. Hanazato & Yasuno (1989) observed the zooplankton community in a pond treated with the insecticide carbaryl. After application all Cladocera and Chaoboridae died. The Chaoboridae, however, quickly recovered and prevented the recovery of Cladocera by their high predation pressure. In a field bioassay, *Chaoborus* may thus be indicative of the occurrence of indirect effects.

An effect on one species, however, does not necessarily represent an effect on taxonomically related species. In the case of duckweeds a pronounced difference in sensitivity to diquat was found between *L. gibba* and *L. minor*.

Our field research was aimed at developing bioassays at various functional levels within an ecosystem. When the bioassays that were developed with the primary producers *Lemna* spp. and *Spirodela polyrhiza*, the carnivorous *C. crystallinus*, and the detritivorous *Gammarus* spp. are performed simultaneously, the results may provide a basis for predictions at higher levels of organization. This approach may be valuable as a supplement to the current risk assessment procedure, which is based mainly on laboratory toxicity data combined with PECs.

In the terrestrial environment the plant species *Poa annua* and *Brassica napus* are chosen as common, but probably sensitive species. Studies of Marrs *et al.* (1989, 1991a) show that there are relatively large differences in sensitivity among species. Our own study with only two species gives the same indication. The ecological function primary production is therefore less likely to be threatened. Such effects as changes in species composition in the direction of less sensitive species are to be expected, however. A study by Marrs *et al.* (1991b) in the field in microcosms confirms this effect.

P. brassicae is an r-strategist, one consequence of which is that in a short period large numbers of caterpillars emerge to eat a lot of food (Bink, 1992). Suppression of this species can therefore lead to food chain disturbance (as intended inside agricultural parcels). The caterpillars also form a food source for a number of organisms such as birds, mammals and predatory invertebrates (Feltwell, 1982). Comparative studies on sensitivity to diflubenzuron show that among the caterpillars *P. brassicae* is one of the most sensitive species (Grosscurt, 1978). Compared to a number of coleoptera species, *P. brassicae* is as sensitive or more sensitive, and the use of *P. brassicae* in bioassays will therefore protect other species. If this is true for other insecticides as well is unclear, however.

In the case of litterbags it will be clear that any effects will be indicative for an ecological function. Since effects were not validated, however, no certainty exist about these effects. The results of the experiment with soil fungi, however, indicate that there may be effects on species composition.

Recovery of effects

In order to judge recovery from effects it is important to have knowledge not only on recovery from a single dose, but also on the frequency with which a compound is applied. In our bioassay studies pesticides were always applied as a single dose. In the bioassays

with duckweeds and litterbags, recovery from effects was observed, even within the relatively short period of the trial. However, in practice there will be more than one treatment, e.g. in the case of captan in fruit, which is sprayed every two weeks. In this case there may be no time for recovery. In most cases, however, no recovery was found in the bioassays.

Ecological relevance of effects

Another question that arises is how should the intensity of an observed effect be valued? In a number of cases effects were statistically significant but relatively small. In this cases the question is: is 5% mortality or growth inhibition ecologically relevant? No general answer to this question can be given. A 5% effect may be entirely swamped by natural variation or recovery mechanisms. On the other hand, a small effect may have an influence on competition between species and lead to relatively large effects on species composition.

Distance of effects

In some cases effects were found at distances where no deposition could be measured. There are two conceivable causes for this phenomenon: i) there was droplet deposition, but below the sensitivity range of the water-sensitive strips; ii) there was no droplet deposition, but vapour drift, which cannot be detected by the water-sensitive strips. Breeze & West (1987a & b) have demonstrated that very low concentrations of airborne pesticides can lead to effects. Eagle (1982) has demonstrated that relatively brief exposure to vapour drift was already sufficient to cause damage. It is therefore probable that vapour drift may play a part, especially in the case of the vascular plants. In a number of cases, however, at 8 or 16 metres' distance very small droplets could be seen on the water-sensitive paper. These droplets were sometimes too small to be detected, however.

Effects were found up to 16 metres downwind of the treated parcels. It is unclear how these results relate to the experiments with untreated field margins. In these experiments (De Snoo, 1994) there was a considerable increase in natural values in 3 to 6 metre wide field margins left unsprayed. Based on the results of the present study, it cannot be excluded that some species in these field margins will suffer from the effects of low deposition rates, or that very sensitive species will not occur in the margins.

5.2 Conclusions

The research questions of this study were:

1. **Is it possible to trace the direct and indirect side-effects of pesticides in the field?**

The results have shown that it is possible to trace direct toxic side-effects outside the target area by means of bioassays. Bioassays are not the most suitable method for tracing indirect side-effects. However, by appropriate selection of test species representative of the various ecological functions, results can be interpreted at an ecological level.

2. What method is most suitable for tracing these side-effects?

The use of bioassays is a suitable and simple method for tracing the direct toxic side-effects of pesticides in the field. In this study, no other methods have been assessed. However, the bioassay method was chosen because it was possible to work under the most controlled conditions in a real field situation.

3. Is it possible to develop standardized field trials for pre- and post-registration?

The use of bioassays forms a basis for developing standard field trials, though for toxic side-effects only. Bioassays can be used for assessing the side-effects of a new pesticide, before legislation, under highly controlled conditions. After registration, side-effects can be assessed by means of bioassays in the practical field situation, where other stress factors also operate.

In addition, the results indicate that pesticide side-effects are to be expected near treated parcels. In most field trials effects have been found 4 metres downwind of a treated plot. In a number of trials effects were found at 8 or even 16 metres' distance. Given the area of agricultural land in the Netherlands, it can be concluded that a considerable area is influenced by pesticide drift.

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