FIELD BIOASSAYS FOR SIDE-EFFECTS OF PESTICIDES
- PROGRESS REPORT -

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FOREWORD AND ACKNOWLEDGMENTS

This report presents the state of the art after two years of a four-year field study aimed at developing field trials for the side-effects of pesticides. Annual progress reports are available in Dutch.

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 en J.A. van Haasteren (both 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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SUMMARY

In 1991 a four-year field study on the use of bioassays to test for side-effects of pesticides was started, commissioned by the Dutch environment ministry. The aim of this study is:

to design, validate and standardize field trials for evaluating toxic and ecological side-effects of pesticides in the aquatic and the terrestrial environment.

In the first two years the use of field bioassay methods was tested employing a variety of organisms with different ecological functions. Tests were carried out on and near treated plots, and the results compared to untreated plots.

In the aquatic environment preliminary investigations were carried out with benthic algae (primary production), water fleas *Daphnia magna*, water snails *Lymnea stagnalis* (herbivores), sticklebacks *Gasterosteus aculeatus* and larva of midges *Chaoborus* (carnivores) and of *Chironomus riparius* (herbivores/decomposers), isopods, amphipods and American River Weed *Elodea spp.* (decomposition). The results were found to vary and in the next two years the study will focus on bioassays with benthic algae, *Lemna spp.*, *Chaoborus* and *Gammarus spp.*.

In the terrestrial environment tests were carried out with Oilseed Rape *Brassica napus* (primary producers), caterpillars of the Large White Butterfly *Pieris brassicae* (herbivores) and litterbags with Rape leaf *Brassica oleracea* discs (representing decomposition). The results were promising and the experiments will be continued and elaborated to produce test protocols.

1 INTRODUCTION

1.1 Background and motivation

In the Netherlands, annual agricultural pesticide use stands at about 22 x 10^6 kg active ingredient (a.i.) (MJP-G, 1991), amounting to an average of 14 kg ha^{-1}. A major proportion (20%-40%) of this mass enters the atmosphere (MJP-G, 1991), either directly during application, as a result of drift, or later, as a result of volatilization from crops and the soil surface. A smaller portion (3%-10%) leaches to ground or surface water. Inside the treated plots as well in the surrounding area the pesticides can contact non-target organisms; occurrence of side-effects (negative effects on non-target organisms) is therefore extremely likely.

Since 1986 CML has conducted studies on 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 studied.

The main result of these studies is that, in spite of the legislation procedure, side-effects are to be expected. At present many standard laboratory tests are available, and 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 laboratory to field led to proposals for designing field trials (De Jong *et al.*, 1990) based on desk studies. As a follow-up of these desk studies, in 1991 the ministry of environment commissioned CML to conduct a four-year field study to investigate the scope for field trials. Halfway through this study this report presents the results to date.

1.2 Objective and problem formulation

The aim of this study is:

> **To design, validate and standardize field trials for evaluating toxic and ecological 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 toxic and ecological 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?**

1.3 Method

Bioassays
The following possibilities exist 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 to the laboratory for studying the effects there. 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 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.

Test species selection
The selection of test species is extremely important for interpreting results (assessing side-effects). In making a choice, test organisms have been selected that represent 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 and indicate 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.

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 results of this selection procedure will be discussed in the chapters on aquatic and terrestrial bioassays, respectively.

Set-up of the field study
In 1991 the field study started with bioassays in the aquatic environment. 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. The methods and results of the aquatic studies are presented in Chapter 2.

In 1991 only a few preliminary experiments were carried out in the terrestrial environment. In 1992 three bioassays were developed. The methods and results of the terrestrial studies are presented in Chapter 3. In Chapter 4, the results are discussed and conclusions drawn. Plans for follow-up research are also presented.

2. AQUATIC BIOASSAYS

In this chapter the experimental set-up of the aquatic studies is first described (§ 2.1). In § 2.2 the results are presented, starting with an overview of the studies carried out in 1991 (§ 2.2.1), followed by a presentation of the results in 1992 (§ 2.2.2) for primary production (§ 2.2.2.1), herbivores (§ 2.2.2.2), carnivores (§ 2.2.2.3) and decomposition (§ 2.2.2.4).

2.1 Materials and methods

Field lay-out

In the vicinity of Leiden, drainage ditches bordering on different crops were investigated. For unexposed control, ditches at a minimum distance of 100 metres were used. The depth of the ditches varied between 20 and 40 cm. For maximum exposed control (1992) pots with test organisms were placed in the crop and compared to pots placed near the unexposed control ditches. The field lay-out generally used is shown in Figure 2.1. For each species tested, four units were placed every five metres. Cropping systems investigated included flower bulb (1991), tree nursery (1991 & 1992), potato (1992), maize (1992) and orchards (1992).

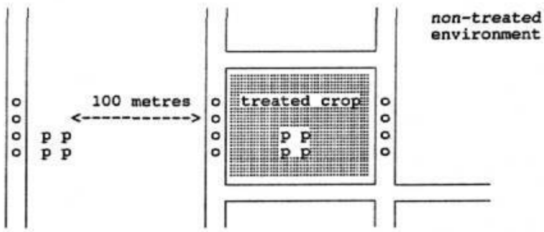

Figure 2.1 Field lay-out for aquatic studies (o = unit with test organisms in the ditch; p = treated control pots)

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 selected test species are summarized in Table 2.1. Selection criteria were common appearance in the habitat studied, functional importance of the species in ditch ecosystems, availability of rearing methods, availability of *in situ* bioassay methods and sensitivity to insecticides.

In situ bioassays

For studying periphyton production, 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 vertically in glass racks and wrapped with steel mesh (1 mm), to avoid grazing by herbivores. In the field the glass racks were fixed in a vertical gauze cylinder (1 mm mesh), in order to keep the water surface free of duckweed and filamentous algae.

Table 2.1 Selected test species

	1991	1992
PRIMARY PRODUCTION	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.

Invertebrates and decomposition of *Elodea* were tested in exposure chambers consisting of a glass jar with a stainless-steel wire screen (0.28 mm mesh for *D. magna*; 1 mm mesh for other invertebrates) in the screw-top lid. When floated, the glass compartment had an air bubble, keeping the wire screen directed downward. The exposure chambers held approx. 250 ml of ditch water. This method has been successfully tested with *D. magna* (ZWO 1990). The exposure chambers for *Asellus* spp., *C. riparius* and decomposition of *Elodea* were placed on the ditch bed. For decomposition of *Elodea*, additional holes measuring approx. 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 40 x 25 x 25 cm. The cages were placed on the ditch bed with the top extending to the water surface, thus allowing the animals to move to the surface under low oxygen conditions. The properties, quantities and responses of the test organisms are summarized in Table 2.2.

In the periphyton bioassay phosphate and nitrate were also analysed at regular intervals. In the bioassay with *D. magna* the chlorophyll-a content of the ditch water was analyzed (NEN 6520) at regular intervals as a measure of food availability. In the bioassay with *C. aculeatus*, oxygen was measured twice daily: in the afternoon and at sunrise.

Laboratory bioassays & toxicity testing

In order to explain and support the results of the field bioassays, in 1992 laboratory bioassays were carried out with *D. magna*. Water samples were taken from ditches to the laboratory and tests carried out in glass jars containing 250 ml of ditch water. Survival was recorded after one week. The medium was tested in duplo.

In 1992 a toxicity test was carried out with *C. crystallinus* and parathion-methyl to support the results of field experiments. The room temperature was 20°C. Glass jars were filled with 0.5 l water from a natural, unpolluted pond. The following concentrations of parathion-methyl were added to the water: 0.1, 1, 10, 100, 1000 μg/l and a control. In each jar 20 animals were tested. For 7 days, survival and behaviour were observed daily.

At the start and end of the experiment dissolved oxygen was measured; the saturation level exceeded 90%. Concentrations were tested in duplo.

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

TEST ORGANISM	STAGE TESTED	ORIGIN	QUANTITY PER UNIT	RESPONSES (TIME)
periphyton algae	-	field	2 glass plates[e]	chlorophyll-a[g] (4 weeks)
D. magna	neonates (0-2 days)	laboratory[a]	10 (1991) 20 (1992)	survival, size, number of eggs and neonates (1 week)
L. stagnalis	egg (3-4 days)	laboratory[b]	1	hatching (4 weeks)
	juvenile (3 weeks)	laboratory[b]	10	survival (4 weeks)
G. aculeatus	adult	laboratory[c]	3 ♀ or 1 ♂[f]	survival, fresh weight (2 weeks)
C. crystallinus	3rd/4th instar	field	10	survival (1 week)
Asellus spp.	adult	field	10	survival (2 weeks)
Gammarus spp.	adult	field	10	survival (2 weeks)
C. riparius	2nd instar	laboratory[d]	10	survival (2 weeks)
decomposition of *Elodea*	-	field	1.5 gr dried substrate	dry weight loss (4 weeks)

[a]culturing method based on NPR 6503, using a 2.5 g/l sea salt solution. 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]size: 40 cm^2 each
[f]at each point 3 cages with 1 ♂ and 1 cage with 3 ♀ were used
[g]according to NEN 6520

Treated control bioassays

In 1992, positive control bioassays with ten specimens of *D. magna* and *C. crystallinus* per jar, filled with 500 ml water, were performed in the fruit crop with the fungicide captan and in the tree nursery with the organo-phosphorous insecticide parathion-methyl, using 4 replicates. Decomposition of *Elodea* was also investigated in the orchard with captan, using 1.5 grams dry weight in 500 ml water, and 6 replicates.

Pesticide concentration estimates & analysis

Pesticide spraying dosages were indicated by the cooperating farmers. For estimating deposition in the bordering ditches, the wind speed and direction at the time of spraying were provided by the Dutch meteorological service KNMI.

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 parathion-methyl. In 1992, the fungicide captan was analyzed once at the fruit location in ditch water and in the treated control bioassays (as described above) one day after spraying.

2.2 Results

2.2.1 1991 overview

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 analyzed with MANOVA (multiple analysis of variance), using pesticide treatment and time as independent variables and excluding interaction effects (insufficient data). No effects of the applied pesticides were found. This is indeed to be expected, since only insecticides (acephate, parathion and pirimiphos) and fungicides (maneb, thiophanate, thiram and triadimefon) were applied. From these results is was concluded that further research is required in herbicide-exposed situations.

Daphnia magna
In the bioassay with *D. magna* high mortality was found in unexposed ditches. Low oxygen levels do not seem to be a cause, since *D. magna* can survive very low oxygen levels (Weider & Lampert 1985).

Survival and reproduction were analyzed by means of MANOVA, with time and pesticide treatment as independent variables and excluding interaction effects (insufficient data). No significant effects of pesticide treatments were found. The two cholinesterase-inhibition measurements showed no activity in the ditches.

From these data it is not clear whether there was no exposure to the applied pesticides, or no effects in the ditches, or effects could not be traced due to wide variation in survival, growth and reproduction. It was concluded that more research should be done after variation in untreated situations and after actual exposure of test animals to the applied pesticides.

Gasterosteus aculeatus
During a warm period very high mortality occurred at all sites. Measurements of dissolved oxygen at sunrise during the warm period showed values as low as 0.04 mg dissolved oxygen per litre. From these oxygen measurements and the apparent negative correlation between survival and temperature it was concluded that mortality was caused primarily by oxygen deficiency. Because of this 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 analyzed in separate ANOVA's (analysis of variance) with pesticide treatment as the independent variable. No effects of the applied insecticides were found.

As no cholinesterase-inhibiting activity was found in the ditches (see also results for *D. magna* above), it is not clear whether there was no exposure to the applied pesticides, no effects in the ditches, or effects could not be traced, due to low survival in the warm period. From the sensitivity of the sticklebacks to low oxygen levels it is concluded that this species is not suitable for use in *in situ* bioassays.

Table 2.3 Summary of results in 1991

IN SITU BIOASSAY	EVALUATION OF METHOD	EFFECTS OF PESTICIDES
periphyton algae	suitable	no effects of insecticides (acephate, parathion, pirimiphos) and fungicides (thiophanate, thiram, triadimefon) found
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 >90%	not tested
Gasterosteus aculeatus	survival very low during warm period, due to low oxygen levels	effects of insecticides during warm period could not be investigated; no effects of insecticides (acephate, parathion, pirimiphos) and a fungicide (thiram) found during cool period
Asellus spp.	survival variable, due to oxygen deficiency	not tested
Gammarus spp.	survival >90%	not tested
Chironomus riparius	survival not known, many specimens lost in sediment	not tested

Preliminary studies with *L. stagnalis*, *Asellus* spp., *Gammarus* spp. and *C. riparius*

The 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 specimens of *L. stagnalis* are suitable for use in 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. On the wire screen of the other chambers there was less or no deposit. It is therefore probable that mortality occurred 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. The conclusion from this experiment is that this species is suited to use in an *in situ* bioassay.

With *C. riparius* the separation of the larvae from the added sediment appeared to be a problem. Only 30% of the animals could be retrieved. Alternatively, another substrate, e.g. glass pearls, can be used in the bioassay. This is less representative of the field situation, however. From this experiment it was concluded that C. riparius is not very suitable for use in an *in situ* bioassay.

2.2.2 1992 studies

2.2.2.1 Primary production: Periphyton

Results

Periphyton production, measured as chlorophyll-a, is presented in Table 2.4. The results were analysed by means of MANOVA, with time and treatments as independent variables, excluding interaction effects (insufficient data). In order to obtain homogeneity of variance, the original data were transformed according to the formula log $(X*1000 + 1)$, where X is the original value. The results shown in Table 2.4 are retransformed values. Prior to the MANOVA's, correlations between chlorophyll-a and phosphate levels in the ditch water were calculated: no correlations were found. In situations where herbicides had been applied, a significant reduction in chlorophyll-a production was found: approx. 65% reduction with diquat/maneb and 35% reduction with atrazine/bentazone. Fungicide treatment alone did not affect chlorophyll-a levels. The maximum pesticide levels in the exposed ditches were estimated to be: 55-385 μg/l maneb, 35 μg/l thiophanate, 50 μg/l pyriphenox, 20 μg/l diquat and 15 μg/l atrazine and bentazone.

Discussion

In pesticide toxicity research with periphyton communities, there is always considerable focus on atrazine. Hamilton *et al.* (1987) and Herman *et al.* (1986) found chlorophyll-a reduction rates of 21% and 68% 35 to 47 days post-treatment with atrazine. These authors, however, used application rates of 80 μg/l and 100 μg/l, respectively. These rates are 5.4 and 6.7 higher than those in our study. 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. In our study, bentazone might also have been a contributing factor to chlorophyll-a reduction. Toxic effects of atrazine on periphyton communities are also found using other parameters, such as carbon uptake (Hamilton *et al.* 1987, Herman *et al.* 1986) and species composition (Hamilton *et al.* 1987, Kosinski 1984). Chlorophyll-a appears to be easier to measure, however.

Table 2.4 Results of *in situ* bioassay with periphyton

CROP	TREATMENT	AVERAGE CHLOROPHYLL-a LEVEL (μg/cm²)	NUMBER OF ANALYSES	95% RANGE (μg/cm²)	EFFECT[a]
potato	- untreated	2.37	32	2.07 - 2.71	
	- maneb (f)	2.34	12	1.85 - 2.95	n.s.
	- diquat (h) /maneb (f)	0.84	4	0.54 - 1.31	p<0.001[b]
maize	- untreated	1.75	19	1.52 - 2.02	
	- atrazine (h) /bentazone (h)	1.14	4	0.79 - 1.66	p<0.05
fruit	- untreated	2.47	8	1.70 - 3.59	
	- thiophanate (f) /pyriphenox (f)	1.88	4	1.11 - 3.18	n.s.

(f) = fungicide; (h) = herbicide

[a]compared to untreated group; n.s. = not significant ($p > 0.05$)

[b]mortality of emergent ditch vegetation, particularly reed, also observed

<u>Conclusion</u>

A reduction of periphyton production was found in ditches exposed to herbicides. These results form a solid basis for further study, and will be validated in the laboratory as well as in controlled field situations (enclosures).

2.2.2.2 Herbivores: *Daphnia magna*

<u>Results</u>

Table 2.5 presents the results of the *in situ* bioassay. Survival in the potato crop was analysed by means of MANOVA, with time and pesticide treatment as independent variables and excluding interaction effects. Because of inhomogeneity of variance, other results were analyzed by means of Kruskal & Wallis analysis of variance with pesticide treatment as the independent variable. No differences were found between treatments, except for effects on reproduction with captan/pyriphenox treatment.

Table 2.5 Results of *in situ* bioassay with *D. magna*

survival

CROP	TREATMENT	AVG. (%)	NUMBER OF CAGES	95% RANGE (%)	EFFECT[a]
potato	- untreated	29.1	119	24.0 - 34.3	
	- maneb (f)	46.5	12	27.9 - 65.2	n.s.
	- dimethoate (i)/cymoxanyl (f) /mancozeb (f)/maneb (f)	49.1	8	26.1 - 72.1	n.s.
maize	- untreated	60.0	58	51.8 - 68.2	
	- atrazine (h)/bentazone (h)	77.5	4	42.5 -112.5	n.s.
fruit	- untreated	32.1	20	19.2 - 45.0	
	- captan (f)/pyriphenox (f)	47.1	4	16.0 - 78.2	n.s.

growth (length)

CROP	TREATMENT	AVG. (mm)	NUMBER OF CAGES	95% RANGE (mm)	EFFECT[a]
potato	- untreated	2.7	78	2.6 - 2.8	
	- maneb (f)	2.7	11	2.4 - 3.0	n.s.
	- dimethoate (i)/cymoxanyl (f) /mancozeb (f)/maneb (f)	3.0	7	2.6 - 3.4	n.s.
maize	- untreated	3.3	50	3.1 - 3.6	
	- atrazine (h)/bentazone (h)	3.7	4	3.0 - 4.5	n.s.
fruit	- untreated	2.9	16	2.7 - 3.1	
	- captan (f)/pyriphenox (f)	2.8	3	2.4 - 3.3	n.s.

reproduction (number of eggs + neonates per survivor)

CROP	TREATMENT	AVG. (number)	NUMBER OF CAGES	95% RANGE (number)	EFFECT[a]
potato	- untreated	1.5	85	1.0 - 2.1	
	- maneb (f)	1.6	11	-0.3 - 4.4	n.s.
	- dimethoate (i)/cymoxanyl (f) /mancozeb (f)/maneb (f)	2.0	7	-0.2 - 3.3	n.s.
maize	- untreated	5.2	50	3.7 - 6.7	
	- atrazine (h)/bentazone (h)	5.9	4	0.5 -11.3	n.s.
fruit	- untreated	1.1	16	0.8 - 1.4	
	- captan (f)/pyriphenox (f)	0.4	3	-0.3 - 1.1	$p < 0.05$

(f) = fungicide; (i) = insecticide; (h) = herbicide
[a]compared to untreated group; n.s. = not significant ($p > 0.05$)

Survival in untreated situations was rather low, especially at the potato and fruit location. In an attempt to find the reason for this high mortality, the influence of ditch water pH and adaptation time of the test animals to the ditch water were investigated. No differences were found between animals directly transferred to the ditch water and animals transferred to the ditch water after 20 minutes to 50% laboratory medium - 50% ditch water. On a sunny day at noon, ditch water pH varied between ditches, ranging from 7.4 to 9.1. However, these values lie well between the limits of tolerance for this species (Frear & Boyd 1967).

In most cases, survival in the water samples taken weekly from exposed and control ditches to the laboratory was higher than 80%. No differences were found between exposed and control ditches. This indicates that pesticide levels in the ditch water at the time of sampling were not toxic to the test animals.

For captan (fruit) and parathion (tree nursery) a treated control experiment was carried out. The results are presented in Table 2.6. *D. magna* showed little toxicity to captan at a level of 1.4 μg/l (measured 24 hours post-treatment). With parathion, applied to the jars by direct spraying at a rate of 240 g/ha, high toxicity was observed, however. At this dosage the level in the treated control was estimated to be approx. 200 μg/l. In the laboratory, toxicity was also found in a 10 times diluted water sample from the parathion-treated control.

Table 2.6 Results of treated controls with *D. magna*

PESTICIDE / CROP	TREATMENT	SURVIVAL (%) AVG. ± S.E. (n)
captan (f) / fruit	+ (1,4 μg/l)[a] - (< 0,04 μg/l)[a]	37 ± 11 (6) 58 ± 11 (6)
parathion (i) / tree nursery	+ -	0 (4) 93 ± 2 (4)

(f) = fungicide; (i) = insecticide; n = number of jars
[a]measured one day post-treatment

Conclusion

After two years of field study 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, because of the lack of control over survival of the test animals in untreated situations. The results also indicate that it is indeed possible to test the toxicity of water from the field. However, the results from such laboratory bioassays also give rise problems of interpretation for field situations. In the further development of field tests, therefore, this species will no longer be investigated.

2.2.2.3 Carnivores: *Chaoborus crystallinus*

Results

In an *in situ* bioassay, one preliminary test was carried out at the tree nursery location. During the test period no pesticide were sprayed. 90% to 100% of the individuals survived after one week.

For captan (fruit) and parathion (tree nursery) a treated control experiment was carried out. The results are presented in Table 2.7. As can be seen, captan showed virtually no toxicity to *Chaoborus* at a level of 1.4 µg/l (measured 24 hours post-treatment). Parathion, applied to the jars by direct spraying, resulting in an estimated concentration of approx. 200 µg/l (cf. results for treated controls with *Daphnia*), showed a high toxicity to *Chaoborus*. In the laboratory, toxicity was also found in a 100 times diluted water sample from the parathion-treated control. This indicates that *C. crystallinus* is more sensitive to parathion than *D. magna* (cf. results for *D. magna*).

In the laboratory the results for parathion were further validated in a dose-response experiment. In this experiment the LC_{50} value at 7 days was 1.05 µg/l; the EC_{50} for behaviour was 0.74 µg/l, which is only 30% lower than the LC_{50} value. These results are in agreement with the observed effect of parathion in the treated control experiment. This value is also comparable to levels applied for pest control: for control of *C. astictopus* in a lake in California, a concentration of 3.3 µg/l was used (Apperson *et al.* 1976).

Table 2.7 Results of treated controls with *C. crystallinus*

PESTICIDE / CROP	TREATMENT	SURVIVAL (%) AVG. ± S.E. (n)
captan (f) / fruit	+ (1,4 µg/l)[a] - (< 0,04 µg/l)[a]	90 ± 4 (2) 100 (2)
parathion (i) / tree nursery	+ -	0 (4) 83 ± 5 (4)

(f) = fungicide; (i) = insecticide; n = number of jars
[a]measured one day post-treatment

Conclusion

The results show that *Chaoborus crystallinus* can be kept well alive in an *in situ* bioassay and that this species is sensitive to parathion, a commonly used insecticide. It is therefore concluded that *C. crystallinus* is potentially suitable as a test organism. This species will consequently be further investigated and applied in an *in situ* bioassay and supportive laboratory bioassays and toxicity tests.

2.2.2.4 Decomposition: *Elodea* as a substrate

Results

In ditches this bioassay has only undergone preliminary testing. In bioassays carried out at the potato and maize location, infestation of the substrate with invertebrates became 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 were analyzed by means of ANOVA. There was no significant difference in dry weight loss, although there was a slight difference between the average values for the treated (51.2%) and untreated group (57.0%). However, visual observation indicated a clear difference in decomposition rate between treatments. Apparently, dry weight loss is not a sensitive parameter for characterizing effects on decomposition.

Conclusion

In the experiment methodic 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* substrate. This method will not be tested further.

3. TERRESTRIAL BIOASSAYS

In this chapter the materials and methods of the terrestrial studies are presented (§ 3.1). The results of the preliminary experiments in 1991 are presented in § 3.2.1 and the results and conclusions of the more extended bioassays in 1992 with primary producers, herbivores and decomposers in § 3.2.2.

3.1 Materials and methods

Field lay-out

The field trials were carried out in the vicinity of Leiden. Unexposed controls were situated at least 200 m from the treated plot. The field situation generally used is shown in Fig. 3.1. Actual commercial application of a pesticide was investigated.

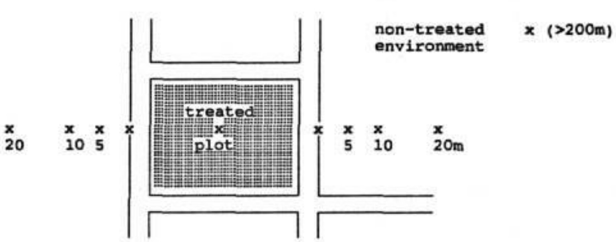

Figure 3.1 Field lay-out for terrestrial studies (x = bioassay)

Test species

The criteria employed for selecting test species have been discussed in the introduction (Chapter 1). For the terrestrial environment we selected the species presented in Table 3.1.

Table 3.1 Selected test species

PRIMARY PRODUCTION	oilseed rape *Brassica napus* 1991: *Lactuca sativa, Raphanus sativus*
HERBIVORES	caterpillar *Pieris brassicae*
CARNIVORES	-
DECOMPOSITION	litterbags

Oilseed rape *Brassica napus* was chosen because it is easily grown, is common in the Netherlands (van der Meijden *et al.* 1983) and is sensitive to herbicides (Eagle 1982).

The Great White butterfly *Pieris brassicae* was chosen for the same reasons, in this case being sensitive to insecticides (Sinha 1989, Van Halder 1991). For assessing effects on decomposition litterbags were chosen, following Heath *et al.* (1964, 1966). In the available time it has not yet been possible to develop a bioassay with carnivores.

Cropping systems and pesticides

The cropping systems and pesticides investigated are presented in Table 3.2. In 1991 only a few preliminary experiment were carried out with *Lactuca sativa* and *Raphanus sativus* as primary producers and litterbags with cellulose paper for decomposition. In 1992 a far more extended research programme was started.

Table 3.2 Cropping systems, pesticides and bioassays investigated

CROPPING SYSTEM	PESTICIDE	BIOASSAY	YEAR
potato	diquat, buminafos	*Lactuca sativa*, *Raphanus sativus*	1991
potato	maneb	*Brassica napus* litterbags *Pieris brassicae*	1992
potato	maneb/diquat	litterbags	1992
maize	atrazine, bentazone	*Brassica napus* *Pieris brassicae*	1992
orchard	captan	*Brassica napus* *Pieris brassicae* litterbags	1992
tree nursery	metam sodium	litterbags	1991
tree nursery	acephate	*Brassica napus* *Pieris brassicae* litterbags	1992
tree nursery	parathion	litterbags	1992
bulb growing	dichloropropane	litterbags	1991
bulb growing	glyphosate	litterbags	1992
bulb growing	dichloropropane/ metam sodium	litterbags	1992

Primary producers: *Brassica napus*

Studies by Marrs *et al.* (1991) demonstrate the feasibility of investigating the side-effects of pesticide drift in the field by means of *in situ* bioassays. In an analogous approach, in 1991 one preliminary experiment was carried out. Because of the season, the experiments were carried out with Lettuce *Lactuca sativa* and Black Radish *Raphanus sativus*.

Bioassay units were placed in and around a potato field treated with diquat and buminafos (leaf-killing herbicides).

In 1992 the test was carried out with Oilseed Rape *Brassica napus*. Each bioassay unit contained 9 potted plants in separate 9 cm pots with potting soil (Fig. 3.2). In this way is was also possible to measure root weight.

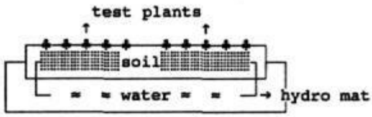

Figure 3.2 Bioassay with vascular plants

Plants were grown in the open air and transferred to the field one day before pesticide application. Six days after application the bioassay units were taken into the laboratory for measuring the wet and dry weight of sprouts and roots. The results were tested with a one-way analysis of variance.

Herbivores: *Pieris brassicae*

Pesticide side-effects on herbivores were studied using the caterpillars of the Great White butterfly *Pieris brassicae* with Chinese Cabbage *Brassica oleracea* as a substrate. Samples consisted of two potted plants; the number of caterpillars varied from two to eight depending on the quantity of food plant at the start of the experiment (Table 3.4). The plants were covered with 1 mm gauze. After one week the bioassay units were taken into the laboratory and survival measured; caterpillars were followed until metamorphosis and the number of butterflies counted. The results were tested using a Kruskal-Wallis analysis of variance.

Decomposition: litterbags

For the litterbag method we followed the method described by Heath *et al.* (1964, 1966). In 1991, two preliminary experiments were carried out, using 20 discs (cellulose paper, 2.5 cm ø) per litterbag as a substrate. The litterbags were made of nylon (25 μm mesh) and measured 20 x 20 cm. In this year, the litterbags were placed in a tree nursery and a bulb-growing field.

In 1992, per litterbag we used 20 leaf discs of Chinese Cabbage *Brassica oleracea*, dried for three hours at 100°C. Compared with cellulose paper these leaf discs have the advantage of being more natural and decomposing faster (*cf.* Heath *et al.* 1964). The litterbags were placed in the field and covered with 1 cm of potting soil. After one week the dry weight of the leaf discs was determined. Litterbags were placed in treated and untreated plots, at 5, 10, 20 and > 400 m distance from the treated plot. Treated plots were represented by agricultural plots treated with the recommended dose of maneb (3 kg a.i./ha) in potatoes, captan (1.5 kg a.i./ha) in fruit and parathion-methyl (0.24 kg a.i./ha) and acephate (1.0 kg a.i./ha) in the tree nursery. In one case the concentration of captan in the soil covering the litterbags was measured 24 hours after application. The results were analyzed by means of ANOVA (analysis of variance).

3.2 Results

3.2.1 1991 overview

In 1991 preliminary experiments were carried out with primary producers and with litterbags.

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

Decomposition: litterbags
After 2.5 months, decomposition in treated plots was less than in untreated plots. This experiment showed that differences in decomposition due to pesticides can be traced using litterbags. Decomposition of cellulose paper was relatively slow, however.

3.2.2 1992 studies

3.2.2.1 Primary producers: *Brassica napus*

Results
The results (Table 3.3) show that in one case of herbicide use (atrazine/bentazone) a side-effect on plants near the treated plot was found. Within the plot, the directly exposed plants all died. Outside the plot, differences in plant size could be seen and measured as plant biomass. This result is shown in Fig. 3.3. In another case an effect was found only within the treated field. No effects of fungicides were found. In the case of acephate a positive effect was found. This appeared to be due to herbivory in the non-treated plant, however.

Sprout wet and dry weight were highly correlated. Root wet weight was very variable and root dry weight reflected the same trend as the sprout. It can be concluded that sprout wet weight alone gives a good indication of effects on plant growth, at least for the pesticides used.

Variation in the 9 plants was such that a difference of only 30% between differently exposed units could be traced with 95% statistical reliability. In the meantime this problem is being remedied; variation has been decreased by growing test plants under more controlled conditions and using larger numbers of plants. A preliminary indoor experiment with *Poa annua* using glyphosate showed a clear correlation between concentration and effects on plant growth.

Table 3.3 Results with oilseed rape in 1992

CROP/PESTICIDE	NUMBER OF UNITS	EFFECT	REMARKS
HERBICIDES			
maize/atrazine, bentazone	10	yes	negative correlation with distance found
maize/atrazine, bentazone	10	yes	effect on exposed bioassay unit only
FUNGICIDES			
potato/maneb	10	no	
potato/maneb	4	*no*	*differences between bioassay units*
fruit/captan	10	no	differences between bioassay units
fruit/captan	4	no	
INSECTICIDES			
tree nursery/acephate	4	yes	positive effect

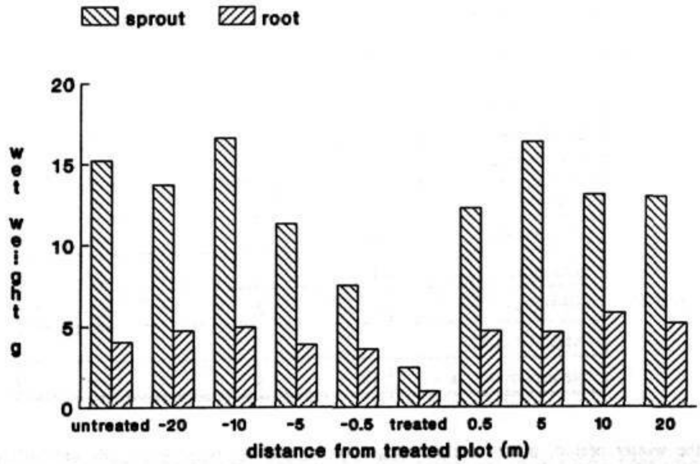

Figure 3.3 Sprout and root wet weight on either side of a atrazine/bentazone-treated maize plot

Conclusions

With respect to the bioassay with vascular plants the following conclusions can be drawn:

- it is practical and easy to carry out;
- test results indicate that side-effects of herbicides can be traced outside the treated plot;
- no side-effects of fungicides could be traced;
- in one case use of an insecticide had a positive effect on plant growth, possibly due to the absence of herbivores following insecticide treatment.

3.2.2.2 Herbivores: *Pieris brassicae*

Results

The results (Table 3.4) show that herbicide treatment was found to have no effects. Although there was a visible effect on the food plants, caterpillars could pupate. Only in one case was a fungicide found to have an effect; here, however, there were large differences between replicates.

In only one case was an insecticide used, with a clear effect on the directly exposed samples. Outside the treated plot, too, many caterpillars died. However, in the non-treated situation some caterpillars also died or escaped. The results of this experiment are shown in Fig. 3.4.

Table 3.4 Results with *Pieris brassicae*

CROP/PESTICIDE	NUMBER OF UNITS	CATERPILLAR/ PLANT	EFFECT
HERBICIDES			
maize/atrazine,bentazone	10	2	no
FUNGICIDES			
potato/maneb	10	4	no
potato/maneb	4	8	yes
fruit/captan	10	8	no
fruit/captan	4	6	no
INSECTICIDES			
tree nursery/acephate	10	4	yes

In the winter period, indoor experiments were carried out with pirimicarb and diflubenzuron and one field trial with diflubenzuron was carried out in 1993. The results show a strong correlation between concentration and caterpillar survival. An effect on the time of pupation was also found. In the field trial there was no survival 2 metres from the treated plot. At 4 metres, a significant effect was also found. At 8 and 16 metres, there were also differences from the untreated plot. These differences were not statistically significant, however.

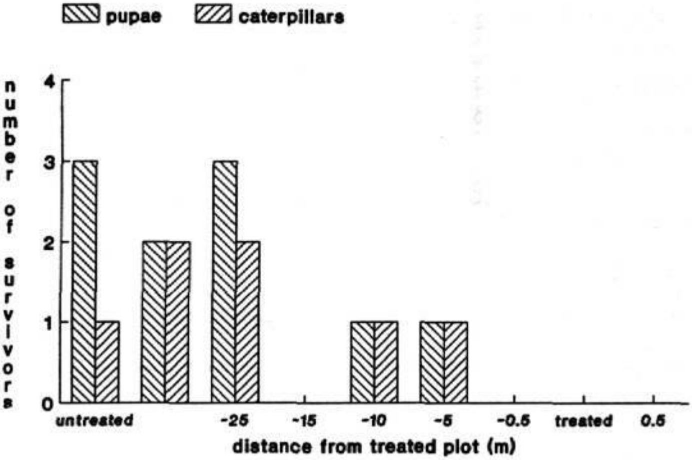

Figure 3.4 Survival of *Pieris brassicae* at tree nursery after acephate treatment

The method will be improved by using larger food plants and more test organisms. Comparable research by Davis *et al.* (1991a & b, 1993) shows that side-effects of insecticides can be traced using caterpillars of the Great White butterfly. The method will therefore be improved to provide a sound base for a herbivore field trial.

Conclusions

The following conclusions can be drawn:

- caterpillars of *Pieris brassicae* can be used for bioassay research;
- preliminary results show indications of side-effects of insecticides outside the treated plot.

3.2.2.3 Decomposition: litterbags

Results

Negative effects on decomposition were found after application of two fungicides (captan and maneb) and an insecticide (parathion-methyl) ($P < 0.05$). The results are shown in Table 3.5. With the insecticide acephate, only one of the two experiments yielded a difference between the treated and untreated plot. In the cases of the fungicides, negative

Table 3.5 Results with litterbags in 1992

CROP/PESTICIDES	SAMPLES	EFFECT	REMARKS
HERBICIDES			
bulb growing/glyphosate	4	no	all vegetation killed
FUNGICIDES			
fruit/captan	10	no	too many variables
fruit/captan	10	yes	correlation found
potato/maneb	4	yes	
potato/maneb,diquat	10	yes	correlation found
bulb growing/dd metam sodium	6	no	treatment two months earlier
INSECTICIDES			
tree nursery/acephate	4	no	variation in litterbags
tree nursery/parathion	4	yes	

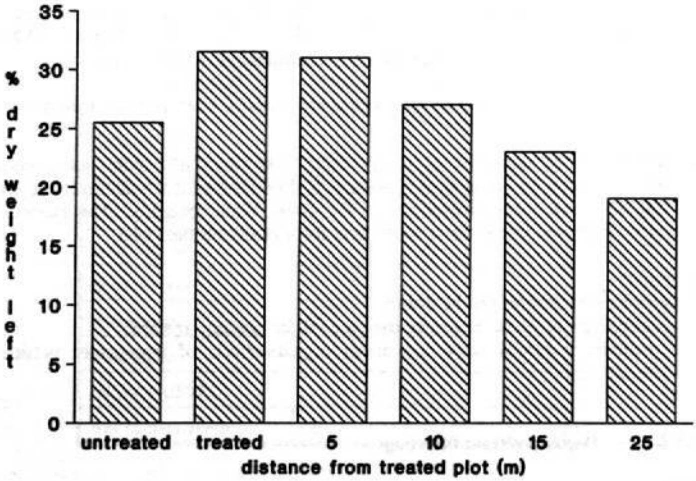

Figure 3.5 Decomposition after one week at various distances from a maneb-treated potato field

effects on decomposition were found up to 10 m from the treated plot. In the range 0 to 20 m from the treated plot, the decomposition rate (70% to 85% dry weight loss) was positively correlated (captan: $R=0.57$, maneb: $R=0.56$, $P<0.01$) with the distance from the treated plot. A negative correlation was found between captan concentration and decomposition ($R=-0.62$, $P<0.01$). The results are shown in Figs. 3.5 and 3.6.

In indoor experiments the results could be validated. The differences were relatively small ($\leq$ 10%), however, and lasted only 6-9 days post-treatment. Further attention will therefore be paid to the biological significance of these results. The relatively small differences are likely to be due to the litterbags being covered with potting soil, making it likely that only a small amount of pesticides reached the bags. In follow-up studies other types of soil will be used (sand).

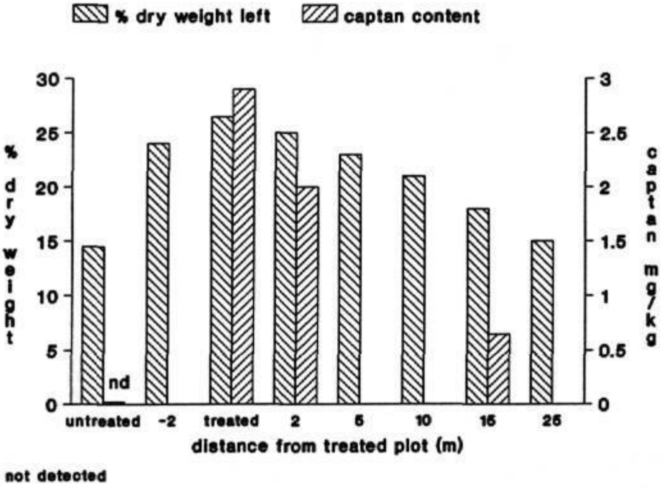

Figure 3.6 Decomposition after one week at distance of a captan treated orchard

<u>Conclusions</u>
From the results it is concluded that:
- the use of litterbags is a quick and simple method for assessing the side-effects of pesticides on decomposition;
- the side-effects of a fungicide could be traced up to 20 m from the treated plot.

4. DISCUSSION, CONCLUSIONS & RECOMMENDATIONS

4.1 Discussion

The aim of these field studies on pesticide side-effects is to provide a scientific basis for protocols for field trials to be used in the legislation procedure (pre- or post-registration). Half way through this field study programme, selection and development of field trails for toxic side-effects are progressing well.

In the case of the aquatic bioassays, the relation between pesticide use and effects is still unclear. One of the main problems is the lack of a treated control situation in the ditches, making it unclear whether non-observance of side-effects is due to the absence of such effects or to the bioassay method not being sensitive enough. In the follow-up of the study, treated controls will therefore be created by means of enclosures.

The terrestrial bioassays have already been standardized to a certain extent. The results show that protocols will probably be able to be developed. Due attention will have to be devoted to interpretation of the test results, however. With vascular plants, the effects on biomass provide a direct indication of a side-effect on primary production. In the case of caterpillars of *Pieris brassicae*, effects have been found on the survival of individuals. The results with litterbags represent a direct effect on decomposition. This effect appears to be relatively minor and of short duration, however. Further research on this duration and on the organisms causing the differences in decomposition will form part of the bioassay study in 1993 and 1994. Among other things, sand will be used to cover the litterbags instead of potting soil.

4.2 Conclusions

At this point we repeat the research questions:

1. Is it possible to trace toxic and ecological side-effects of pesticides in the field?

The results have shown that it is possible to trace toxic side-effects outside the target area by means of bioassays. Bioassays are not the most suitable method for tracing ecological 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 toxic side-effects of pesticides in the field.

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 only for toxic side-effects. 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.

4.3 Follow-up studies

Aquatic studies

For 1993 and 1994, the aquatic part of the research will be concerned with enclosure studies. In polder ditches, compartments will be created where various bioassay units can be exposed to controlled quantities of a pesticide. With this set-up, it will be possible to include a treated control in the experiments. The development of bioassays with *Chaoborus* and periphytic algae will be continued. Furthermore, bioassay methods will be developed with Duckweed *Lemna spp.* and *Gammarus*.

At a later stage of the study, enclosures will be used near a treated plot. In this case deposition will be measured using water-sensitive paper and concentration measurements will also be made.

Terrestrial studies

To protect experiments from the sometimes unpredictable behaviour of the farmers, in 1993 the terrestrial bioassays will be conducted in a controlled experimental plot. In this way treatments can be precisely planned and favourable weather conditions waited for.

The terrestrial bioassays have already been standardized to a certain extent. It therefore seems possible to formulate preliminary protocols for these tests.

The litterbags will be studied in more detail. Further study will focus on the processes and organisms causing the differences in decomposition in the litterbags.

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