Effects of the Pesticides Esfenvalerate and Prochloraz on Pond Ecology

Pesticides Research

An insecticide (Sumialpha) and a fungicide (Sportak) are investigated for different effects. Growth inhibitions of Asellus aquaticus were especially elucidated. Growth inhibitions are seen at 9.75 ng/l for Sumialpha. Furthermore, Sumialpha has acute effects on behaviour and surface activity on surface animals in the used test doses.

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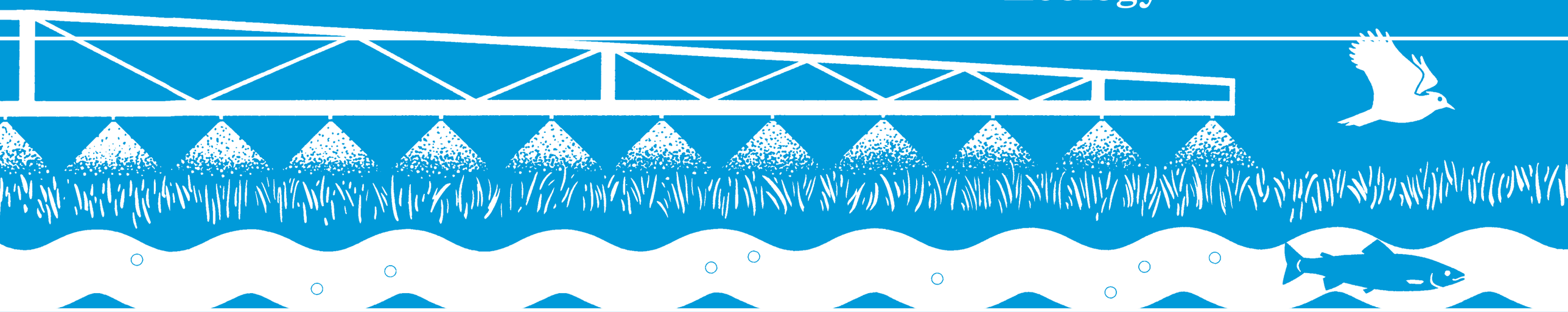

Ministry of Environment and Energy, Denmark
Danish Environmental Protection Agency

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Effects of the Pesticides Esfenvalerate and Prochloraz on Pond Ecology

With special attention on Asellus Aquaticus (L)

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Ministry of Environment and Energy, Denmark
Danish Environmental Protection Agency

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Foreword

This report it is a part of the research programme "Effects of Pesticides on Ponds", funded by the Pesticide Office at the Danish Environmental Protection Agency (DEPA). This program has been undertaken by four institutions in various degrees of co-operation: Amphiconsult (as a consultant for the regional council of Funen), Danish Technological Institute (DTI), The National Environmental Research Institute (DMU) and The Department of Environment, Technology and Social Studies, Roskilde University. The program was supervised by a steering committee with Mr Jens Mossin (DEPA) as chairman and representatives from the participating institutions. External representatives were Mr Arne Schøitz, director of the Danish Aquarium and Associate Professor Per Rosenkilde, Institute of August Krogh, Copenhagen University.

Each of the participating institutions had their own subprojects. Amphiconsult examined the pesticide impact on amphibians in natural ponds. DTI analysed the level of pesticides in natural waters and accomplished LC_{50} tests on amphibian larvae. DMU investigated the fate of pesticides in the sediments of artificial ponds. The Department of Environment, Technology and Social Studies examined the effect of pesticides on invertebrates in the laboratory and in artificial ponds (mesocosms).

At the same time, the report is a part of a Ph.D. study undertaken at the Department of Environment, Technology and Social Studies, Roskilde University with Associate Professor Henning Schroll as supervisor.

I thank Associate Professors Per Homann Jespersen and Henning Schroll for reading, writing, offering comments about the manuscript and for helping with the statistical analyses. I thank Stine Bohr and the technical staff of the department for various technical assistance's. Thanks to the participants in the steering committee for being a source of inspiration, Per Rosenkilde, Lars Briggs, Henning Klausen, Claus Hansen, Peter Wiberg-Larsen Betty Bügel Mogensen and Jens Mossin.

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Summary

Asellus aquaticus as indicator

The author has studied two selected pesticides effects on pond ecology, with special attention to *Asellus aquaticus*. The aim of the project was to improve the knowledge of sublethal effects of the two pesticides on *Asellus aquaticus*, and to study the direct and indirect effects on macroinvertebrates and periphyton in mesocosms.

Artificial ponds (mesocosms)

Six artificial ponds (mesocosms) were established of which four were used in experiments. A Laboratory with an air-conditioning plant was established in order to control temperature conditions in experiments with *Asellus aquaticus* in aquaria.

Two pesticides

Two pesticides were selected in the study based on toxicity and frequency of application. One was the insecticide, Sumialpha®, containing the active ingredient esfenvalerate. The other was Sportak®, a fungicide containing the active ingredient prochloraz.

Asellus aquaticus was chosen as indicator and experimental species because of its wide distribution and frequency in both lentic and lotic environments. In addition, *Asellus aquaticus* is easily kept in laboratory.

Laboratory experiments

For **Sumialpha,®** the estimated $EC50^{48h}$ for *Asellus aquaticus* of 2.5 ng/l (nominal) changed significantly when organic material was added. The $EC50^{48h}$ when organic matter was added were 110 ng/l (nominal). Both Sumialpha® and technical grade esfenvalerate affected the growth rate of *Asellus aquaticus*. Sumialpha®, the commercial product and hence the ecologically more significant, had the greatest effect. A significant effect on the growth rate in dry weight and length for small and medium-sized animals was found at 9.75 - 18.75 ng/l (nominal). For technical grade esfenvalerate no effects were measured on the chosen concentrations on medium-sized animals, but an effect on growth rates in dry weight on small *Asellus aquaticus* were found at 37.75 ng/l (nominal).

Sportak® had no effect on the growth rate for the chosen concentrations (max 560 µg/l, small and medium sized animals; 1120 µg/l, big animals) but mortality increased significantly by increasing concentrations. $EC50^{48h}$ was estimated to be 665 µg/l (nominal).

Mesocosm experiment, Sumialpha®

Sumialpha® reduced the density of larvae and nymph of insects and *Asellus aquaticus* at the lowest concentration. The lowest concentration (38 ng/l nominal) is equivalent to an application directly on the pond surface by 1/16 of recommended dose. Gastropoda and Lammellibranchia showed no response but the results are uncertain. Oligochaeta had no acute respond in density, but a significant increase in density over time.

Cage studies, meant to test the validity of the extrapolation from laboratory data to field conditions, failed because of high mortality.

Sumialpha® changed the activity and behaviour of surface breathing animals in a dose-response manner. The behavioural changes were the lack of escape by disturbance plus flight out of the water (crawling on seashore).

Periphyton biomass decreased in the reference pond and increased in the treated ponds. This may be interpreted as an indirect effect caused by the elimination of some grazers.

Fish in two experimental mesocosms

Fish were observed during the experiment with Sumialpha® and after the first period of experiment, perches were fished out of two mesocosms by Electro-fishing. In one mesocosm was an overcrowded community of 80 perches, in the other was 8 perches. It was estimated that the overcrowded community had a significant influence on the fauna composition, why it is not used in the interpretations of effects. For the other mesocosm, it is estimated that there is no significant effect from fish.

Mesocosm experiment, Sportak®

It was not possible to determine if **Sportak®** had an effect on the density of animals in the treated ponds, many groups being only present in low numbers. The lowest concentration was 8 µg/l (nominal) and equivalate to an application of one half of the recommended dose directly on the pond surface.

In cage studies, no significant changes in mortality or growth rate were measured.

No significant change in surface activity or behaviour was observed, but mortality increased.

The periphyton biomass decreased in the treated ponds, with the lowest biomass in the pond with the highest concentration treatment. The decrease in biomass is interpreted as a direct toxic effect.

Dansk sammendrag

Asellus aquaticus som indikator art

Under Miljøstyrelsens bekæmpelsesmiddelsforskningsprogram "Effects of Pesticides on Ponds" startede i 1994 denne undersøgelse af to udvalgte pesticiders effekter på vandhulsøkologien, med ferskvandsbænkbideren *Asellus aquaticus* som indikator art. Formålet med projektet har været at skaffe viden om 2 pesticiders subletale effekt (vækst) *på Asellus aquaticus* samt at undersøge direkte og indirekte effekter på makroinvertebrater og periphyton i kunstige vandhuller (mesokosmos).

Kunstige vandhuller

Der blev udgravet 6 kunstige vandhuller, hvoraf de fire blev brugt i forsøg. Klimarum blev etableret i laboratoriet, for at skabe ensartede forhold under akvarieforsøg med *Asellus aquaticus*.

To pesticider

To pesticider blev udvalgt til at indgå i undersøgelsen på baggrund af toksicitet og anvendelseshyppighed. Det var et insekticid, Sumialpha®, med det aktive stof esfenvalerat, samt et fungicid, Sportak®, med det aktive stof prochloraz.

Asellus aquaticus blev valgt som indikator- og forsøgsdyr på baggrund af artens udbredelse og hyppighed i såvel vandhuller som vandløb. Desuden er arten let at holde i laboratorium.

Laboratorieforsøg

For **Sumialpha®** viste forsøg i laboratoriet, at den estimerede $EC50^{48h}$ for *Asellus aquaticus* på 2,5 ng/l ændrede sig til 110 ng/l (nominel) ved tilsætning af organisk materiale i akvarierne. Endvidere havde både Sumialpha® og teknisk rent esfenvalerat en effekt på vækstraten hos *Asellus aquaticus*. Effekten var størst med Sumialpha®, der er handelsproduktet og derfor det økotoksitokologisk mest relevante. Der blev målt en effekt på vækstraten i tørvægt og længdevækst for små og mellem store *Asellus* fra 9,75 ng/l - 18,75 ng/l (nominel). For teknisk rent esfenvalerat sås ingen effekt på mellemstore *Asellus* ved de valgte koncentrationer, men en effekt på vækstraten i tørvægt på små *Asellus* fra 37,75 ng/l (nominel).

Sportak® viste ingen effekt på vækstraten ved de valgte koncentrationer (maks 560 µg/l for små og mellemstore, 1120 µg/l (nominel) for store Asellus), men mortaliteten steg signifikant med koncentrationen. $EC50^{48h}$ er estimeret til 665 µg/l (nominel).

Mesokosmos forsøg, Sumialpha®

Sumialpha® reducerede densiteten af larver og nymfer af insekter samt *Asellus aquaticus* ved den laveste koncentration. Den laveste koncentration på 38 ng/l (nominel) svarer til en oversprøjtning af vandhullet med en 1/16 af anbefalet dosis. Der var ingen effekt på snegle og muslinger, men resultaterne er usikre. Børsteormene viste ingen akut respons, men havde en kraftig densitets forøgelse over tid.

Burforsøg, der skulle teste validiteten af ekstrapolation af den laboratorie fundne effekt, mislykkedes pga. for stor dødelighed.

Sumialpha® havde en dosis-respons indvirkning på overflade åndende dyrs aktivitet og på adfærden. Overfladeaktiviteten steg mest i det højest behandlede vandhul. Adfærds ændringer var manglende flugtreaktion ved forstyrrelse, samt flugt ud af vandet (kravlende på bredden).

Der var et fald i periphyton biomassen i referencevandhullet og en stigning af biomassen i de behandlede vandhuller. Dette kan tolkes som en indirekte effekt, ved elimination af visse periphytongræssere i de behandlede vandhuller.

Fisk i to forsøgsvandhuller

Under forsøget med Sumialpha®, blev observeret fisk og efter den første forsøgsperiode blev der ved elektrobefiskning opfisket aborre fra to forsøgsvandhuller. I den ene var der et tusindbrødre samfund på 80 små aborre, i det andet var der kun 9 individer. Det estimeredes at aborre havde en betydende effekt på faunaen i vandhuller med tusindebrødre-samfundet, hvorfor det ikke indgår i tolkninger af effekter. I det andet vandhul er det estimeret, at der ikke er betydende effekt fra fisk.

Mesokosmosforsøg, Sportak®

Det kunne ikke afgøres om **Sportak®** havde en effekt på densiteten af dyr i de behandlede vandhuller, mange grupper var kun sparsomt repræsenteret. Den laveste koncentration (8 µg/l nominel) svarede til en oversprøjtning af vandhullet med halvdelen af anbefalet dosis.

Burforsøgene viste ingen signifikant forskel i mortalitet eller effekt på vækstraten hos *Asellus aquaticus*.

Der var ingen ændringer i adfærd hos overfladeåndende dyr, men en øget mortalitet.

Der var et fald i periphytonbiomassen i de behandlede vandhuller, med den laveste biomasse i det højest behandlede vandhul. Faldet tolkes som en direkte toksisk effekt.

1 Introduction

It is a general assumption that pesticides and nutrients impact ponds and streams in areas where industrialised farming is present. The impact can be caused by direct spraying or wind transportation or as surface or subsurface run off (Day, 1989), see *Figure 1.1*

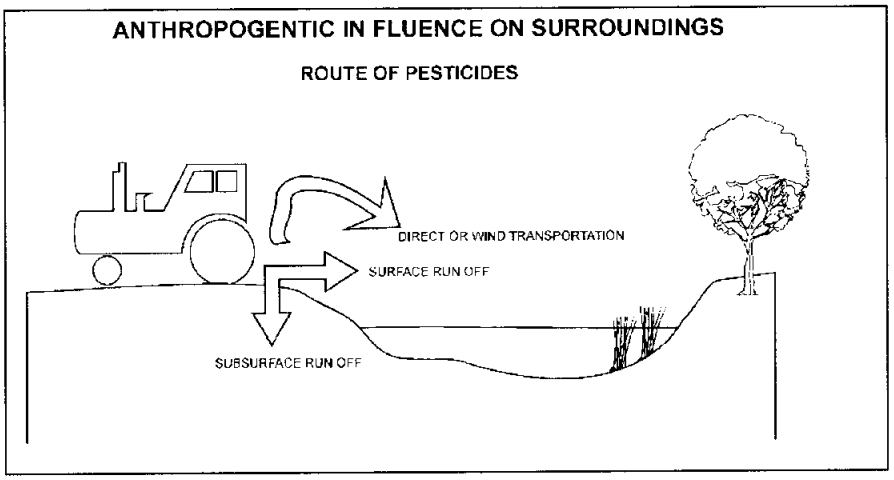


Figure 1.1

Possible routes of pesticides to the aquatic environment.

Mulige veje for tilførelsen af miljø fremmede stoffer til et vandhul.

Several selected pesticides are found in streams (Spliid og Mogensen, 1995). The same report refers to a study in Sweden (Kreuger and Brink, 1988) where relatively high levels of pesticides were measured in streams. It is therefore reasonable to assume that various concentrations of pesticides can be found in Danish aquatic ecosystems.

Natural or artificial toxins are often found in aquatic environments in sublethal concentrations but very little is known about effects on living aquatic organisms (Jones et al., 1991). Even less is known about indirect effects and the influence on the community in the ecosystem, yet these indirect effects might be common. Studies have shown that the herbicide Atrazin have effects in mesocosms not only on plant communities (macrophytes and phytoplankton) but also a sublethal effect on the aquatic fauna (invertebrates, tadpoles and fish) (Neugebaur et al. 1990, Denoyelles et al. 1989, Dewey, 1986).

The objective of this project is to provide information about 2 pesticides concerning:

- Sublethal effects on *Asellus aquaticus* in microcosms (Aquarius)
- To test the extrapolation of sublethal effects on *Asellus aquaticus* from microcosms to mesocosms (artificial ponds)
- Effects on macroinvertebrate density in mesocosms
- Effects on periphyton biomass in mesocosms
- Indirect effects in mesocosms.

In particular three reports provided the background for the practical execution of the project (EWOFFT, 1993; SETAC-EUROPE, 1992; SETAC-RESOLVE, 1992). The reports contain recommendations of investigations of micro- and mesocosms. The recommendations are followed where it has been practically and economically feasible.

The report has five sections.

- A general methodological section
- A study concerning an insecticide (esfenvalerate)
- A study concerning a fungicide (prochloraz)
- Conclusion of the two studies
- Addendum and Appendix

2 Methods

The Steering committee selected an insecticide and a fungicide on the following 3 criteria:

- The pesticide should be very toxic to aquatic organisms.
- The pesticide should be among the five most utilised in farming practices.
- The pesticide should not be expected to be out phased in the near future.

Against this background, the insecticide esfenvalerate and the fungicide prochloraz were selected.

Esfenvalerate

Esfenvalerate is a synthetic pyrethroid. The chemical name is (S)-α-cyano-3-phenoxybenzyl (S)-2-(4-chlorophenyl)-3-methylbutyrate. The molecular formula is $C_{25}H_{22}ClNO_3$. The insecticide kills a wide range of insects. It acts through contact or ingestion (Tomlin, 1995) by blocking the conduction in the synapses of the nerves (Hassall, 1990), although there is a dispute whether it is a direct or indirect effect of the pyrethroid (Clark and Brooks, 1989). The pyrethroids are generally more toxic to aquatic organisms than to terrestrial organisms (Anderson, 1989; Coats et al., 1989; Hill, 1989).

Prochloraz

Prochloraz is an imidazole. The chemical name is N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-l-carboxamide. The molecular formula is $C_{15}H_{16}Cl_3N_3O_2$. The fungicide inhibits the ergosterol biosynthesis (Tomlin, 1995). The substance is a non-systemic (weak systematic) fungicide acting on seed- or earthborn pathogens belonging to Ascomycetes, Deuteromycetes and partly Basidomycetes (Hassall, 1990).

2.1 Laboratory experiments

The Department of Environment, Technology and Social Studies selected *Asellus aquaticus* as an indicator species. It was done based on the following considerations:

- The species should belong to a group of animals that is known to be vulnerable to insecticides and fungicides in fresh water.
- The species should be widespread in Danish ponds.
- Because it is expected to carry out further studies later in running waters the species should have broad ecological amplitude and be found in lotic as well as lentic environments.
- The animal should be tolerant to oxygen depletion (nutrient pollution).
- The animal should be easy to keep in the laboratory.
- The animal should be non-migrating so that each stage of the life cycle can be exposed to pesticides in the aquatic environment.
- The animal should be well described in the literature.

In a monitoring programme in North Zealand *Cloeon* spp. and *Asellus aquaticus* were found in respectively 76% and 66% of the included 33 ponds (Mogens Holmen, personal communication). Both taxa are also found in lotic environments, and they are well described in the literature.

Asellus aquaticus is also very tolerant to oxygen shifts, non-migrating and easy to keep in the laboratory.

Asellus aquaticus as indicator and test animal

Asellus aquaticus fits the criteria well and it was selected as test animal.

Growth experiments and replicates.

Growth rate of the animals was chosen as the sublethal parameter for investigation. For statistical reasons, the growth experiments were carried out in five replicates. Experiments where at least four replicates were significant alike at the 5% level were included. The experiments were not designed to evaluate the mode of effect, that is whether the effect are caused by directly contact or indirectly by feeding organic matters with adsorbed pesticide.

3 sizes of animals

Different sizes of animals were used in the experiments. Small growing animals have a high metabolism and they are more exposed to impact than bigger and older animals. Three sizes of animals were chosen, and a sorting device was constructed in order to provide well defined size groups (see section 2.1.2)

2.1.1 Collecting test animals

As the experimental design should be as close to natural conditions as possible, it was decided to collect test specimens from a natural pond instead of breeding test specimens in the laboratory. In order to secure that animals have the same genetic background, adaptation, and a similar response in the tests, all the test animals were collected from the same location. The choise of a natural pond was obvious, since the laboratory tests should be compared with the mesocosms experiments.

Stodderklemmen

A pond (Stodderklemmen) near RUC was chosen to be the supply of test animals. *Asellus aquaticus* from this pond were used for the initial experiments. Stodderklemmen is surrounded by a cattle run owned by Roskilde municipality and used by the municipality's Nature School. The area was not and has not been treated with pesticides in at least 5 years. All the initial laboratory experiments were carried out with animals from this pond.

Adaptation, preserving and EC_{50}

The initial laboratory tests were used to 1) examine the animals adaptation to the laboratory conditions, 2) find the best way to preserve the animals, and 3) to estimate EC_{50}. Furthermore, the collected animals were used to establish relations between different morphological characters and dry weight or wet weight.

Slagslunde skov

Later Stodderklemmen had to be given up. A combination of a very dry and unusual hot summer and presumably a major supply of nutrient from cows, that used the pond as drinking water, caused an almost completely extinction of animal life in the pond. This caused half a year delay of the laboratory experiments. An alternative pond was found in Slagslunde forest, Esrum forest district, close to Slangerup. The pond is a former peat bog (from the 1940's) surrounded by deciduous forest, mainly alder and beech. A minor spring enters the pond. Pesticides are not used in the surroundings.

Collection of animals

The collections of animals were made with a landing net. The samples were roughly sorted in the field and other animals than *Asellus aquaticus* and plant materials were removed. The samples were washed and transferred to a transportation container. The time of transport was minimized. The samples were kept cold until sorting in the laboratory.

2.1.2 **Sorting procedure**

In the laboratory, the samples were cleaned in a 250 µm sieve. The animals were sorted in three size groups. A sorting device was constructed to make the sorting easier and especially to avoid damage of the animals.

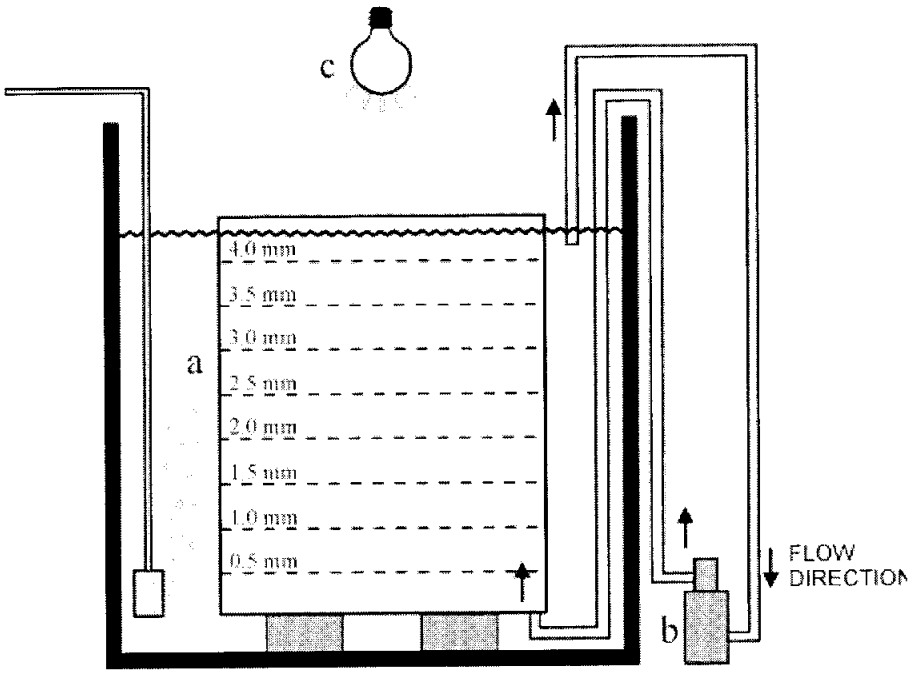


Figure 2.1

Sorting device. a: Stack of boxes with descending sizes of holes, b: Pump, c: Source of light. See text for explanation.

Udsorteringsapparat. a: Stabel med faldende hulstørrelse, b: Pumpe, c: lyskilde. Se tekst for forklaring.

Positive geotaxis, negative rheo- and phototaxis

The device consists of a stack of boxes (a) with well-defined holes in the bottom from 0.5mm to 4.5mm and with 0.5mm interval starting with the biggest holes in the top. Over the aquarium is a source of light. A number of animals are placed on the top of the stack, covered, and placed in the aquarium. A pump (b) sends water current up through the stack. Because of positive geotaxis and negative rheo- and phototaxis the animals will seek down through the stack until the holes are smaller than the width of body. A pilot experiment showed that animals of 5mg wet weight have a body width of approximately 1.6mm. These animals were captured in the stack between 2.0mm and 1.5mm. An outcome of the pilot experiment was a relation between average body width F(X) and wet weight (X):

$$F(X) = 0{,}8889\ln(X) + 0{,}4727$$

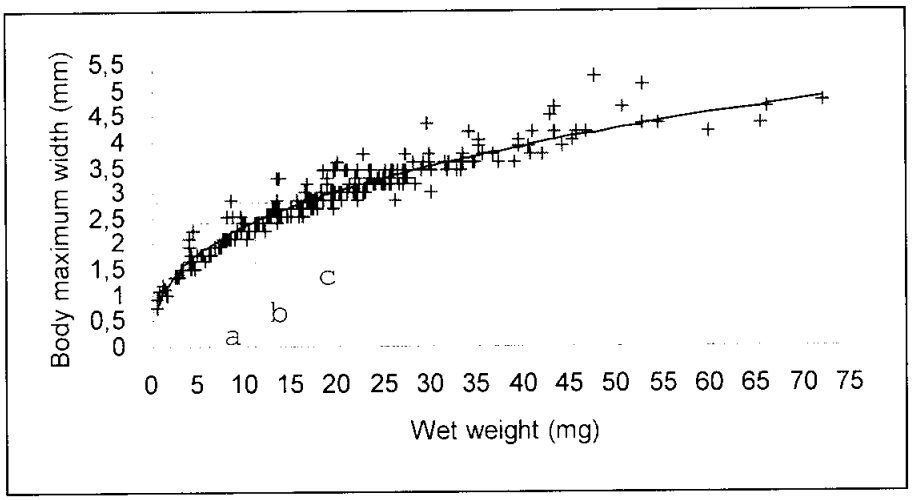


Figure 2.2

Body width / Wet weight relation for Asellus aquaticus, n=250, R^2=0,9394 (Pedersen, 1998). a: small Asellus, b: medium Asellus, c: big Asellus in laboratory experiments.

Kropsbredde / vådvægts relation for Asellus aquaticus, n=250, R^2=0,9394 (Pedersen, 1998). a: små Asellus, b: mellem store Asellus, c: store Asellus i laboratorieforsøg.

The size groups are well separated.

A similar sorting device has successfully been used for *Asellus aquaticus* and Gammarus pulex (Pedersen, 1998; Pedersen, 1990; Larsen and Thylin, 1983).

300 animals from each size group were collected. 50 animals from each size group were taken randomly for weighing and measurement for determining the variance within the group.

2.2 Mesocosms experiments

Originally, the steering group decided that the experiment should be realised using natural ponds. This would necessitate a number of similar ponds with the same geological background. Furthermore, information about the local use of pesticides should be known. In co-operation with Roskilde County, some areas were found in the neighbourhood of Køge and Roskilde. The natural ponds were given up because the county

- Did not want to expose the ponds to pesticide impacts
- Could not find enough suitable ponds.

Objective of mesocosms

The Danish Environmental Protection Agency (DEPA) investigated the possibilities for using an area belonging to the military, but after half a year of investigations the military strategy was given up. In the end, DEPA funded 10,000 Kr. to establish a number of artificial ponds (mesocosms) close to RUC. The objective of these ponds was to examine the impact of

selected pesticides on the growth rate of *Asellus aquaticus* and the abundance of invertebrates in the sediment. Furthermore, the study should evaluate the validity of an extrapolation of laboratory results to more natural conditions.

2.2.1 Establishing the ponds

The ponds were established on a clay rich area of RUC. The territory was found by examining areas for water saturation and by taking soil samples. Two areas were examined by a geo electric method that is able to elucidate soil types in deeper layers with some precision.

Establishing ponds

In the beginning of June 1994, six ponds were excavated by contractor Tom Nielsen. According to the plan each hole should be 12 m^2 (6x2 m. with a gradient of 1:4), a maximal depth of 1m and a volume of 8 m^3. Considering the tools (according to Tom Nielsen) a further gradient of 1:3 was made on the two long sides and the one broad side, which meant, that the length of the pond became 9m and 8m wide. The ponds did not become exactly similar and the depths vary. However, the forecast of a layer of clay was correct and water began to fill the ponds up to 40cm. Unfortunately, the intruding water hindered the holes from being drummed. Until July 1, the ponds were filled with tap water, and the depths varied between 85cm to 105cm. After July 1, no additional water was supplied to the ponds but the water levels were frequently measured. In spite of an exceptional warm and dry summer, the ponds did not dry up but the water level sank steadily at almost the same rate for each pond. This indicates that the change in water level was caused by evaporation and not a sink. The advantage was that the ponds should not be lined with plastic, which could have resulted in contamination with plasticizer and a potential binding of pesticides to the plastic.

In the beginning of September 1994, the ponds were implanted with sediment from Stodderklemmen.

Mesocosms had approximately 13 months to stabilise.

The Roskilde municipal's Nature School provided a tractor and manpower for the transportation of sediment. The sediment was homogenised in a tub and transported to RUC. After the sediment was placed in the ponds, the water levels were raised to approximately 65cm, and from then on allowed to fluctuate between 60cm to 70cm. Water plants such as bur reed (*Sparganium*), reed mace (*Typha*), bulrush (*Scirpus*), tufted loosestrife (*Lysimachia*) and mare's-tail (*Hippuris vulgaris*) were planted. Duckweed and filamentous algae were removed. Organic materials such as alder leaves were added to the ponds. The ponds were allowed to stabilise until August 1995 in order to allow immigration.

The alder leaves were collected in an alder forest close to the city of Lejre. The leaves were distributed evenly on the sediment surface. An area of 1.5 metre x 0.5 metre were marked by sticks, and used as collecting area of benthic invertebrates and surface animals.

The ponds were numbered from 1 to 6 starting from the eastern end. Pond number 1 and 6 were given up as test mesocosms because they dried up rather fast. Pond 1 was made deeper and used as a water reservoir. Pond no.

2, 3, 4 and 5 were used as test mesocosms, while no. 6 was organism and breeding pond.
Different considerations were taken into account concerning the experimental design. The number of ponds was limited because of lack of funding. Six ponds were the compromise and that were sufficient for executing a regression analysis (Setac-Europe, 1992) for three concentrations including two reference ponds and a reservoir. Unfortunately, this model had to be modified because two ponds had to be exempted from the experiment.

2.2.2 **Application of pesticides**

The volumes of the ponds were estimated in order to calculate a nominal concentration of pesticide. The estimation was made to avoid a dose of pesticide that could be expected to be lethal for most of the invertebrates. The pesticide was sprayed by a hand sprayer, and each pond was sprayed five times as to ensure an even distribution. The ponds were sprayed walking continuously around the pond. To avoid drift by the wind, banks surround each pond. Spraying was done on calm days. The ponds were sprayed with a fraction of the recommended dose per m^2 once in the experiment period.

Esfenvalerat: 1/4, 1/8 and 1/16 of recommended doses. Prochloraz: 2/1, 1/1 and ½ of recommended doses.

The nominal concentrations of esfenvalerate were estimated to be respectively 132, 77 and 35 ng/l. The nominal concentrations of prochloraz were estimated to be 34, 19 and 8 µg/l.. Esfenvalerate was sprayed with respectively 1/4, 1/8 and 1/16 of the recommended dose. Prochloraz was sprayed with respectively 2/1, 1/1 and 1/2 of the recommended dose.

2.2.3 **Benthic invertebrates**

Different freshwater organisms appear in the ponds originating from the inoculated sediment or from direct implantation, especially *Asellus aquaticus*. After a period of stabilisation and before the pesticide application the diversity and abundance of invertebrates were examined.

To reduce disturbance as much as possible the animals were collected with a plastic tube with a sampling area of $22cm^2$. An approximately 10cm long column was taken. Ten samples were taken stratified random in each pond each time and within a marked area of 150cm x 50cm (Elliott, 1977).

The area was subdivided in three times ten sections. Ten sections were randomly selected and the sampling started in one end of the area directed against the other end so that each randomly selected sample did not disturb other sampling. The substrate consisted of clayey gravel with organic material (alder leaves) on which water thyme (Elodea canadensis) were growing. Precautions were taken to secure that the sampling area in the ponds had a unvaried substrate. From the depth of 20 centimetre the described sediment substrate was dominating in all ponds and the substrate was in that way representative.

Termination of experiment and endpoints

Population data:
- -Biomass (*Asellus aquaticus*)
- -Abundance

Diversity
Recovery (only esfenvalerate experiment)

The sampling of benthic invertebrates on a uniform substrate has been arranged in order to reduce variety of animals but there will still be a considerable risk of aggregation of benthic animals. The substrate has been manipulated to fit *Asellus aquaticus*, which belongs to the feeding functional group of shredders. In the ponds, *Asellus* will mainly feed on decomposed alder leaves. Other parameters like radiation of light and predation can cause an accumulation of animals. Furthermore, other species than *Asellus* might prefer other types of substrate and in that sense the manipulated substrate is not optimal for sampling other benthic invertebrates in the ponds.

2.2.4 **Surface invertebrates**

Surface invertebrates were collected within a marked area of 1.5m^2 before the pesticide spraying, 1½ hours and 24 hours after the pesticide was sprayed. The net had an opening of 40 cm and the mask size were 500 µm.. The behaviour of the invertebrates as surface activity or other activity was observed.

2.2.5 **Periphytic algae**

The changes in chlorophyll a of periphytic algae were studied on object glasses placed on a stand in the water. Four stands each with 5 object glasses were placed in each pond with the object glasses 10cm below the surface. On four different dates 5 replicates from each pond were collected for measuring the periphytic chlorophyll a. The stands were placed in the ponds 3 weeks before spraying, so that a colonisation of periphytic algae could take place. The samples were analysed in a modified way after Ferskvandsbiologisk laboratorium (Freshwater Biological Laboratory) (1985) and DS2201, because the object glasses were placed in ethanol for the extraction of chlorophyll a. The guidelines in DS2201 was followed.

2.2.6 **Cage experiment**

Short-term experiments were performed in cages. In each pond five cages with 10 *Asellus aquaticus* in each cage were placed. The cages were made of Pyrex glass with a 250 µm net as a cover. Each cage was provided with alder leaves as food. The cages were placed on tiles on the bottom.

Besides being a controlled field test, the cage experiment should test the extrapolation of the laboratory tests to the mesocosms tests.

Termination of experiment and endpoints.

The endpoints are mortality, length and width of the animals, WW, DW, growth, growth rate and fecundity.

2.2.7 **Chemical data**

Before and during the experiment, the temperature was measured continuously with a data logger (Tinytalk-temp), oxygen concentration below the surface and over the bottom with an oxygen electrode, alkalinity (DS 253),

DS: Dansk standard = Danish Standardisation.

nitrate-N with NO_3-electrode and phosphate-P (DS 291). At the beginning of the experiment cadmium (Cd), calcium (Ca), potassium (K), sodium (Na) and silicon (Si) was measured by atomic absorption spectrophotometer (DS 2214 and DS 259).

For the chemical analysis of the pesticide concentration, 1 litre of water was taken 10cm below the surface before and during the experiment. The water samples were immediately frozen for later analysis. The analysis of the pesticide concentration was made by the Section of Chemical Technology, Danish Technological Institute (DTI). The method implies a methylene-chloride extraction of the samples and an analysis by a combined gas chromatographic and mass spectrographic method by the use of selective ion monitoring.

3 The Esfenvalerate experiment – results and discussion

3.1 Laboratory experiments and methodological considerations

Growth tests were performed for two formulations of esfenvalerate.

- One test was prepared with Sumialpha® 5FW which is the trade product containing 5% esfenvalerate. This formulation is used by the farmers.
- Another test used technical pure esfenvalerate dissolved in acetone. Acetone was used as solvent according to a DTI guideline. DTI uses acetone as solvent in their tests, and acetone is a common solvent for pyrethroids (Hassall, 1990 and Tomlin, 1995).

The tradition in toxicology is to use the technical pure active substance in tests. This procedure gives a reliable reference because the formulation of the trade product might vary. However carriers and auxiliaries and other so-called inert ingredients in the trade product can be toxic and might have a synergistic effect (Briggs, 1992). Since the formulated trade product Sumialpha® constitutes the actual environmental impact, it has a high eco-toxicological interest, and it was decided to use the trade product in the study.

3.1.1 Sublethal concentrations and methodological considerations

EC50 was estimated in two different experimental conditions. In the first test (*Figure 3.1*) Asellus was not fed. In the second test (*Figure 3.2*) organic material as conditioned alder leaves were added (5 leaves, weight 691.16mg ± 25.37mg 95% C.L., n=174). Esfenvalerate was added as the formulation Sumialpha®

$EC50^{48h}$ without organic material was calculated to be 2.5ng/l (nominal concentration). $EC50^{48h}$ with organic material was 110ng/l (nominal concentration), see *(Figure 3.1)* and *(Figure 3.2)*. Sumialpha® was approximately 40 times more acutely toxic than when no organic material was added.

The growth experiments were expected to run for 50 days and Asellus would be fed by alder leaves. Against this background, the $EC50^{48h}$ with organic material was used for calculations of the sublethal concentrations to be used in the growth experiments.

Figure 3.1

EC50 experiment using Sumialpha® and Asellus. No feeding using alder leaves. $EC50^{48h}$ approx. 2,5 ng/l . Effects: Immobility or death.

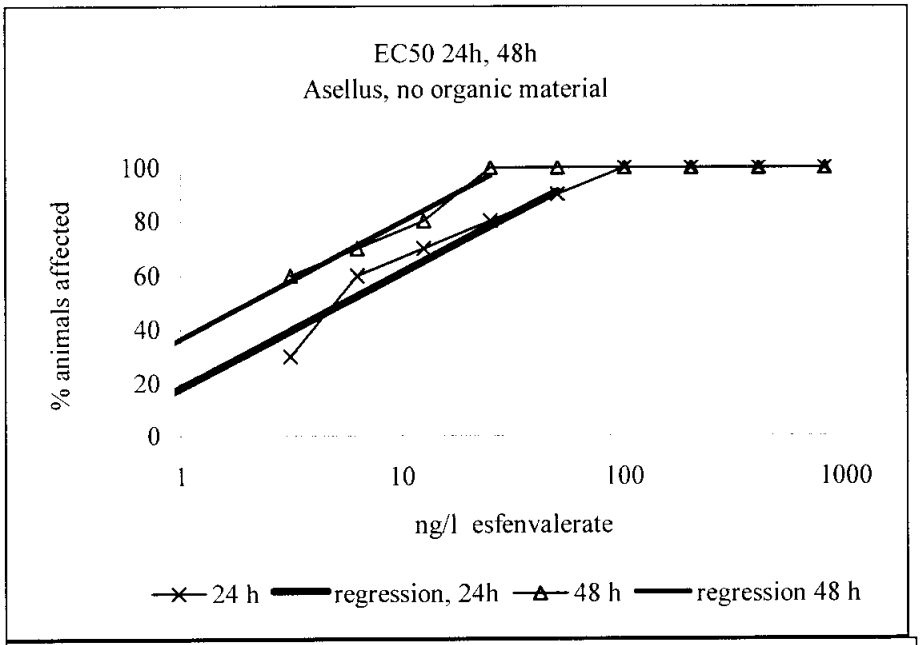
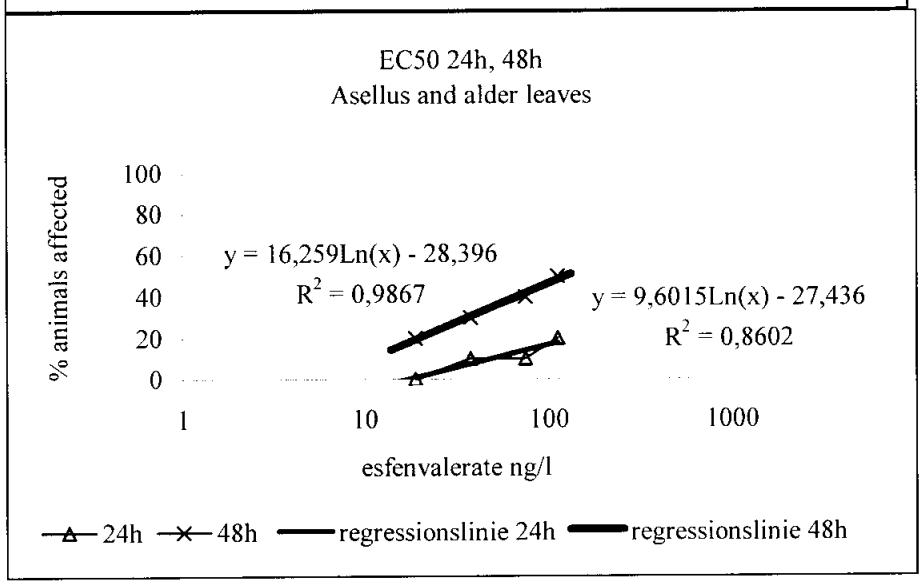


Figure 3.2

EC50 experiment using Sumialpha® and Asellus fed on alder leaves. $EC50^{48h}$ approx. 110ng/l. Effects: Immobility or death.



3.1.2 **Growth experiment with Sumialpha®**

The growth experiments were accomplished after 51 to 57 days in a laboratory air-conditioning plant at 10 °C. 550 *Asellus aquaticus* were used in three size groups with 50 animals distributed on five replicates in each size group and concentration. The concentrations appear from *Tabel 3.1.* The concentrations are nominal concentrations of esfenvalerate. Alder leaves were added (5 leaves 691.16mg ±25.37mg 95% C.L., n=174).

Measured concentrations are shown in the appendix. The measured concentrations are less than nominal concentrations, which would be expected, but the samples were measured 2½ year after the original experiment, why the results are questionable.

Tabel 3.1

Results from growth experiments with Asellus aquaticus (L) in different concentrations of formulated esfenvalerate (Sumialpha®). Small Asellus from hole size 1 - 1.5mm; medium Asellus 1.5 – 2.0mm; big Asellus 2.0 – 2,5mm. n=500

Resultater fra vækstforsøg med Asellus aquaticus (L) i forskellige koncentrationer af Sumialpha®. Små Asellus udsorteret fra hulstørrelse 1 - 1,5 mm; mellem Asellus : 1,5 - 2,0 mm; store Asellus 2,0 - 2,5 mm. n=500.

TREATMENT/ ANIMAL SIZE	Length (mm) ± 95% C.L.	WW (mg) ± 95% C.L.	DW (mg) ± 95% C.L.
SMALL			
initial	6.2 ±0.1	6.47 ±0.48	0.84 ±0.06
control	7.2 ±0.2	13.07 ±1.12	2.79 ±0.30
18.75 ng/l	6.8 ±0.2	12.37 ±1.20	2.44 ±0.31
37.5 ng/l	5.9 ±0.2	8.20 ±0.84	1.54 ±0.19
MEDIUM			
initial	7.1 ±0.2	10.90 ±0.55	1.88 ±0.13
control	8.0 ±0.1	17.48 ±1.05	3.66 ±0.21
9.75 ng/l	7.7 ±0.2	17.34 ±1.68	3.78 ±0.39
18.75 ng/l	7.7 ±0.2	16.12 ±1.26	3.33 ±0.29
75.0 ng/l	7.8 ±0.2	16.73 ±1.21	3.45 ±0.31
BIG			
initial	7.7 ±0.1	15.45 ±0.71	3.11 ±0.16
control	8.1 ±0.2	19.61 ±1.23	4.01 ±0.39
18.75 ng/l	8.2 ±0.3	18.93 ±1.39	3.62 ±0.27
37.5 ng/l	8.4 ±0.3	19.15 ±1.47	3.97 ±0.44

Small animals: Initial end holes size 1.5 mm. Growth experiment 51 days.
Medium animals: Initial end holes size 2.0 mm. Growth experiment 55 days.
Big animals: Initial end holes size 2.5 mm. Growth experiment 57 days.

Concerning the medium size group, the results of the concentration 37.5ng/l have been omitted because the replicates were not similar (ANOVA, $p<0.05$). In this test series the glasses were overgrown with filamentous algae which was not the case with the other glasses in the experiment.

Mortality

The mortality within the size groups and test series were not significant different (ANOVA, p=0.45 (small), p=0.45 (medium), p=0.48 (big)) and were as average for the small animal 20% ± 7, medium size animals 29% ± 9 and big animals 31% ±11. *See table 3.2*

Mortality in % and 95% C.L. in Asellus aquaticus growth experiment with Sumialpha® (see Tabel 3.1). All in five replicates of 10 Asellus aquaticus, n=500.
Small: No significant difference in mortality (ANOVA, p=0.45) Medium: No significant difference in mortality (ANOVA, p=0.31). Big: No significant difference in mortality (ANOVA, p=0.53)

Mortalitet i % med 95% C.L. i Asellus aquaticus vækstforsøg med esfenvalerat formuleret som Sumialpha® (se Tabel 3.1). Alle i 5 replikater af 10 Asellus aquaticus , n=550.
Små: Der er ingen signifikant forskel på mortaliteten i forsøget (ANOVA, p=0.45). Mellem: Der er ingen signifikant forskel på mortaliteten i forsøget (ANOVA, p=0.31). Store: Der er ingen signifikant forskel på mortaliteten i forsøget (ANOVA, p=0.53)

	control	9.75 ng/l	18.75 ng/l	37.75 ng/l	75 ng/l
Small	17 ±11		18 ±13	26 ±12	
Medium	22 ±12	23 ±15	27 ±13	40 ±14	33 ±15
Big	34 ±16		25 ±14	23 ±17	

Specific growth rate

All the animals grew in relation to the starting variables (t-test, all p<0,001), and the growth rate was calculated as the specific growth rate Gs (% per day).

$$Gs = (\ln(\text{end variable/initial variable}) * t^{-1}) * 100\ \%$$

(Sutcliffe et al. 1981).

The specific growth rates for the dry weight (DW) and length appear in *Figure 3.3* and *Figure 3.4*.

Figure 3.3

*The specific growth rate (dry weight) in % per day. n=500 animals. Small Asellus: Growth rate declines significantly*** *with concentration. Medium: Control + 9.75 not significantly different***, 18.75 + 75 not significantly different***. Significant*** decline in growth rate from group 1 to group 2. Big: Not significantly different***.*

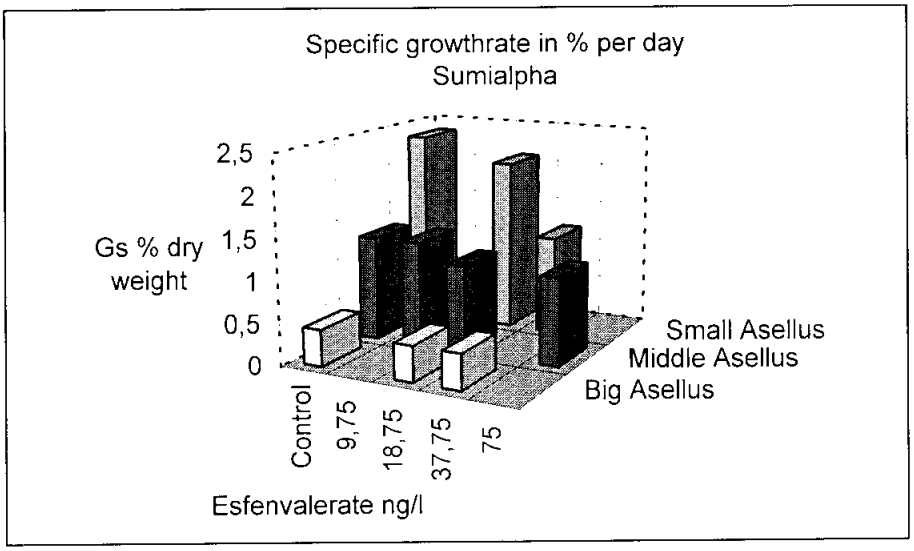
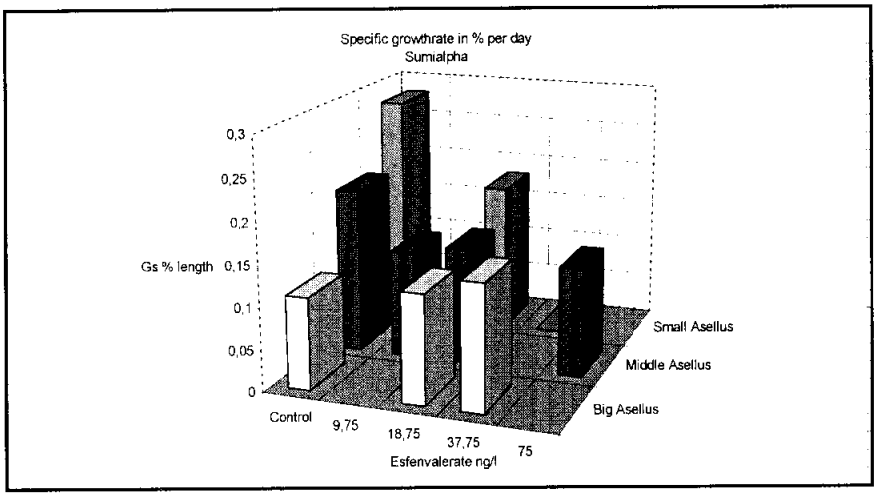


Figure 3.4

*Specific growth rate (length) in % per day. n=500 animals. Small Asellus: Growth rate declines signifiantly*** *by concentration. Medium Asellus 9,75 , 18,75 and 75 not significantly different***, a significant decline in relation to control. Big Asellus: Not significantly diffenrent***.*



From *Tabel 3.1* and *Figure 3.3* and *Figure 3.4* it appears that small animals were more sensible to Sumialpha® compared to big animals for the selected concentrations. The size group "big animals" was not sensible. There is a clear doses/response relation for the small animals while the doses/response relation for medium size animals is not clear although the specific growth rate falls compared to the control.

3.1.3 **Growth experiment using esfenvalerate**

The growth experiments were accomplished after 35 days in a laboratory air-conditioning plant at 10 ^{0}C. 500 *Asellus aquaticus* were used in two size groups with 50 animals distributed on five replicates in each size group and concentration. The concentrations appear in *Table 3.3*. The concentrations are nominal concentrations of esfenvalerate. As an additional control, five replicas with the solvent acetone were included in the experiment. Alder leaves were added (5 leaves 691.16mg ±25.37mg 95% C.L., n=174).

The big size group was not used since the preceding experiment did not show a response.

Table 3.3

Results from growth experiments with Asellus aquaticus (L) in different concentrations of esfenvalerate. Small Asellus from hole size 1-1.5 mm; medium Asellus 1,5 – 2.0 mm. n=500

Resultater fra vækstforsøg med Asellus aquaticus (L) i forskellige koncentrationer af esfenvalerat. Små Asellus udsorteret fra hulstørrelse 1 - 1,5 mm; mellem Asellus : 1,5 - 2,0 mm. n=500.

TREATMENT/ ANIMAL SIZE	Length (mm) ± 95% C.L.	WW (mg) ± 95% C.L.	DW (mg) ± 95% C.L.
SMALL			
initial	5.3 ±0.2	5.21 ±0.43	1.16 ±0.11
control	6.1 ±0.2	8.48 ±0.70	2.03 ±0.19
acetone	6.1 ±0.3	7.99 ±0.99	1.88 ±0.27
18.75 ng/l	6.2 ±0.2	9.13 ±0.66	2.00 ±0.18
37.5 ng/l	6.2 ±0.2	8.37 ±0.78	1.62 ±0.21
75 ng/l	6.2 ±0.2	7.96 ±0.77	1.46 ±0.21
MEDIUM			
initial	6.4 ±0.1	9.74 ±0.49	2.02 ±0.15
control	6.9 ±0.2	12.00 ±1.00	2.30 ±0.25
acetone	6.9 ±0.2	12.45 ±0.94	2.54 ±0.23
18.75 ng/l	6.9 ±0.2	12.25 ±0.93	2.31 ±0.23
37.5 ng/l	6.8 ±0.2	11.86 ±0.94	2.25 ±0.23
75.0 ng/l	6.7 ±0.3	11.15 ±0.94	2.18 ±0.23

Small animals: Initial end holesize 1.5 mm. Growth experiment 35 days.
Medium animals: Initial end holesize 2.0 mm. Growth experiment 35 days.

No glasses or replicates are excluded from the calculations.

Mortality in experiment

The mortality within the size groups and the test series were not significantly different (ANOVA, p=0.06 (small), p= 0.82 (medium), and the average for small animals was 22% ±4 and medium size animals 21% ± 6. The mortality in the tests appears in *Table 3.4.*

Tabel 3.4

Mortality in % and 95% C.L. in growth experiment with Asellus aquaticus and esfenvalerate (see table 3.3). Five replicates of 10 Asellus aquaticus, n=500. Small: No significant difference in mortality (ANOVA, p=0.06). Medium: No significant difference in mortality (ANOVA, p=0.82).

Mortalitet i % med 95% C.L. i Asellus aquaticus vækstforsøg med esfenvalerat (se table 3.3). Alle i 5 replikater af 10 Asellus aquaticus , n=500. Små: Der er ingen signifikant forskel på mortaliteten i forsøget (ANOVA, p=0.06). Mellem: Der er ingen signifikant forskel på mortaliteten i forsøget (ANOVA, p=0.82).

	Control	acetone	18.75 ng/l	37.5 ng/l	75 ng/l
Small	20 ±9	13 ±11	17 ±7	32 ±9	25 ±10
Medium	28 ±14	18 ±16	20 ±16	20 ±16	18 ±17

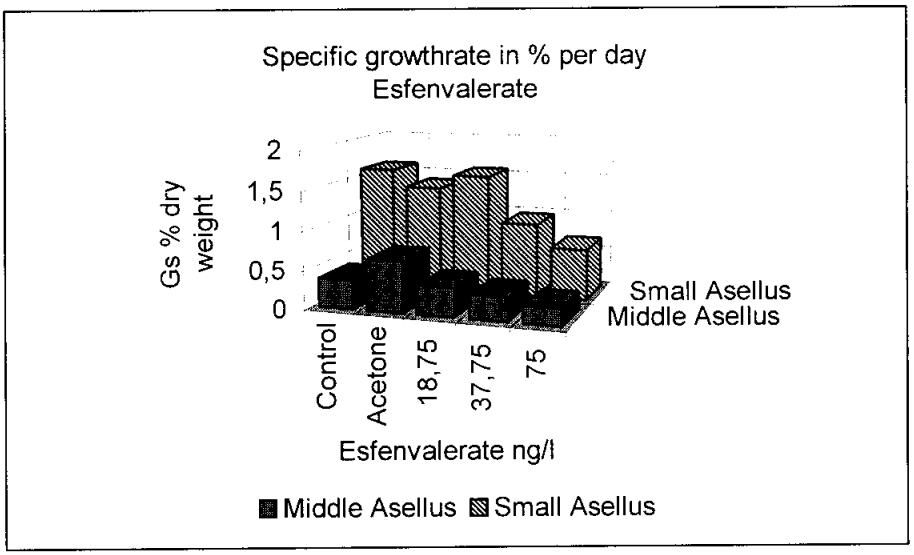
Specific growth rate

All the animals grew compared to the starting conditions (t-test, all p<0,001). The specific growth rate of dry weight (DW) and length appears from *Figure 3.5* and *Figure 3.6.*

.

Figure 3.5

*Specific growth rate (dry weight) in % per day. n=500 animals. Small Asellus: Control, 18.75 and acetone are not significant different; 37.75 and 75 not significant different. Between the two groups are a significant*** *decline. Medium Asellus: No significant difference.*



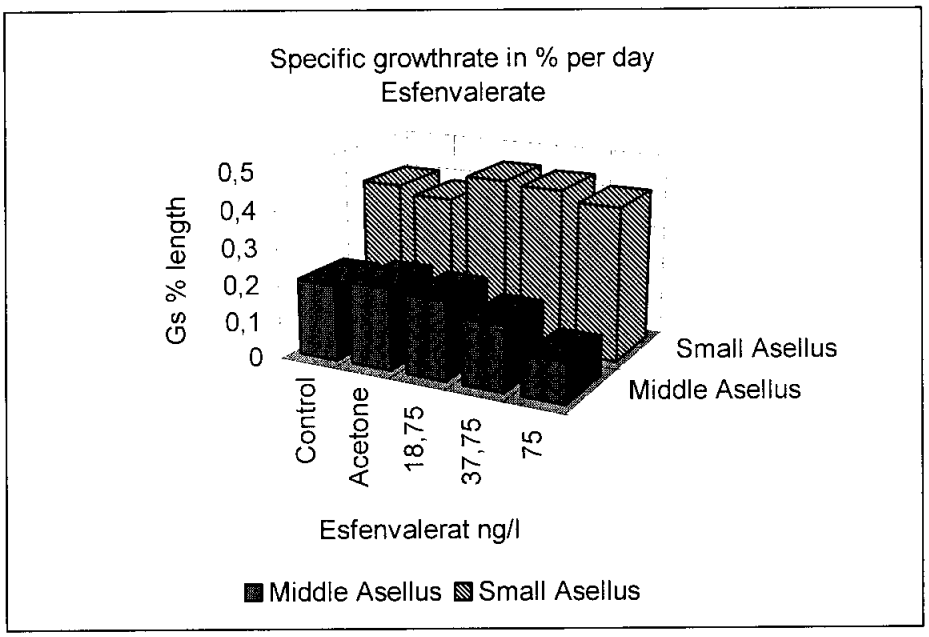


Figure 3.6

*Specific growth rate (length) in % per day. n=500 animals. Small Asellus: No significant difference***. Medium Asellus: No significant difference***.*

The specific growth rate (DW) (*Figure 3.5*) for small animals show more sensibility to esfenvalerate when compared to medium size animals. The medium size animals were not sensitive to esfenvalerate. The growth rate declined for small animals from 37.75ng/l but there was no significant difference in the growth rate from 37.75ng/l to 75ng/l. For the specific growth rate (length) (*Figure 3.6*) there were no significant differences of growth rates for small and medium size animals.

3.1.4 **Summary**

$EC50^{48h}$

$EC50^{48h}$ for *Asellus aquaticus* is significantly different in test with or without organic material. $EC50^{48h}$ is estimated to 110ng/l and 2.5ng/l (nominal esfenvalerate concentrations) respectively.

Growth rate in Sumialpha® experiments

There is a significant effect of Sumialpha® on the growth rate in dry weight and length of small *Asellus aquaticus*. The effect is doses/response related. There is an effect on medium *Asellus aquaticus* and no effect on big Asellus

Growth rate in technical pure esfenvalerate experiments.

An effect of technically pure esfenvalerate on the growth rate of small *Asellus aquaticus* in the dry weight is seen but not on the length. There is no difference in the effect from 37.75ng/l to 75ng/l. There is no effect on medium size *Asellus aquaticus*.

Mortality

No significant differences in mortality appear in the two experiments.

Conclusion

On this basis, it is concluded that Sumialpha® has a higher effect on the growth rate compared to technical pure esfenvalerate. Since Sumialpha® is the formulated product that farmers actually apply in the agricultural production, it seems reasonable to use Sumialpha® in the mesocosms experiments instead of the technical pure esfenvalerate.

3.2 Pond experiments (mesocosms)

The results from the mesocosms are presented in the following. The experiments were started July 31 1995. Experiments with prochloraz started May 7 1996 and the first sample date was used to assess a long-term effect of esfenvalerate.

3.2.1 Chemical-physical data

pH (figure 3.7)

The average pH in the ponds was approximately 8.7 (Figure 3.7) and there were no significant differences between the ponds.

Oxygen (figure 3.8 and figure 3.9)

The surface oxygen was as on average 9.4mg/l (4.8-15.8) (*Figure 3.8*) during the period for all four ponds while the bottom oxygen on an average was 5.3mg/l (0.7 -9.5) (*Figure 3.9*). The oxygen concentrations were lowest in pond 2 and 4.

Temperature (figure 3.10)

The temperature declines during the experiment. To August 25th 1995, the average temperature was 22°C with an average daily fluctuation of ± 2 °C (max. 26°C, min. 17°C). From August 26th 1995 the average temperature falls to 16°C with an average daily fluctuation of ±1 °C (max. 18°C, min. 12°C) (*Figure 3.10*)

Ions (Tabel 3.5)

Measured ions in the water and sediments are shown in Table 3.5.

Sumialpha® (table 3.6)

The chemical analysis of water samples for esfenvalerate after spraying with Sumialpha® are presented in Table 3.6. After 48 hour, the active substance esfenvalerate had disappeared from the surface. It should be noted that the samples were kept in a freezer for 1½ years before the analysis. It is not known what influence the time delay has on the results.

Pond 2 was a reference pond. Pond 5 was sprayed with 1/4 of a normal dose, pond 3 with 1/8 dose, and pond 4 with 1/16 dose.

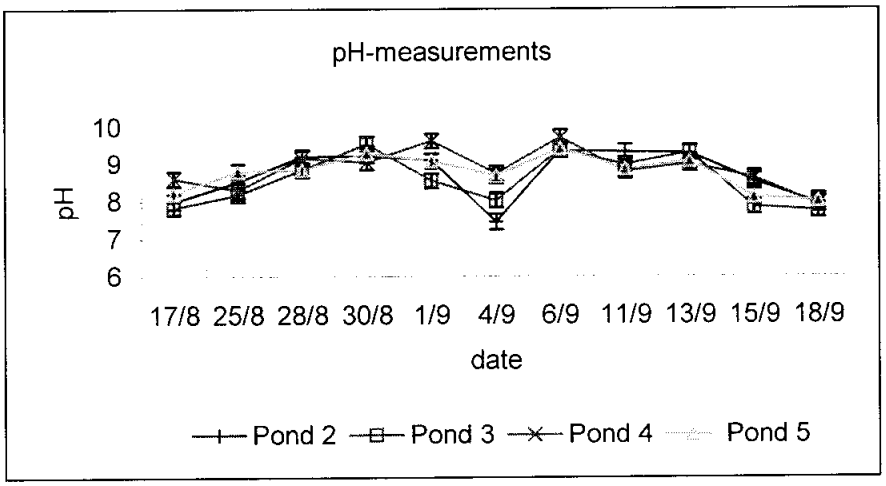


Figure 3.7

pH – measurements. No significant difference in pH in the ponds. Average pH is 8.73 ± 0.18 (95% C.L.).

pH - målinger . Der er ingen signifikant forskel på pH i de 4 vandhuller. Den gennemsnitlige pH er 8.73 ± 0.18 (95% C.L.).

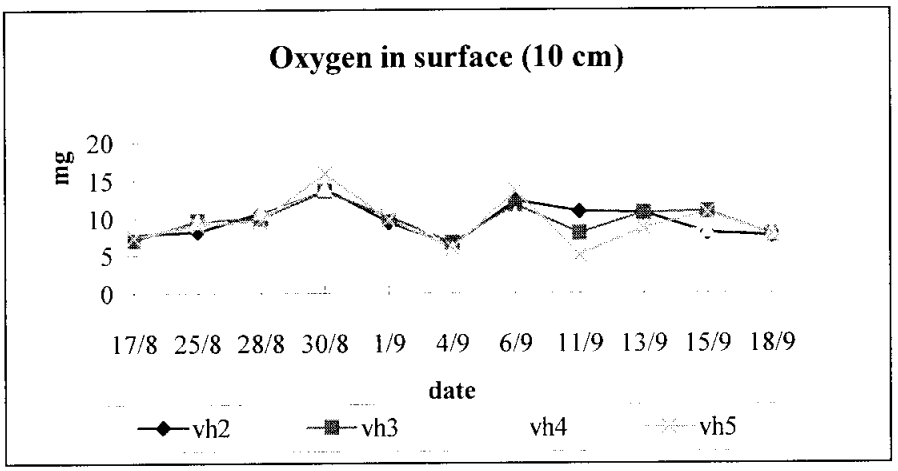


Figure 3.8
Oxygen in mg/l measured 10 cm beneath surface.

Ilt i mg/l målt 10 cm fra overfladen.

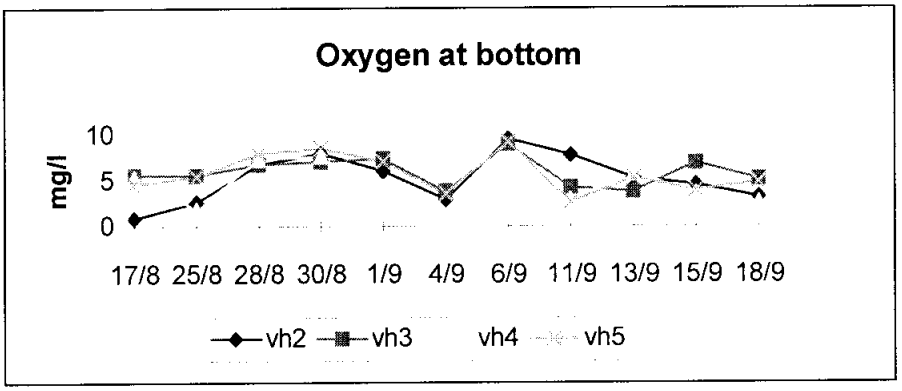


Figure 3.9
Oxygen measured at the bottom, mg/l

Ilt målt ved bunden i mg/l.

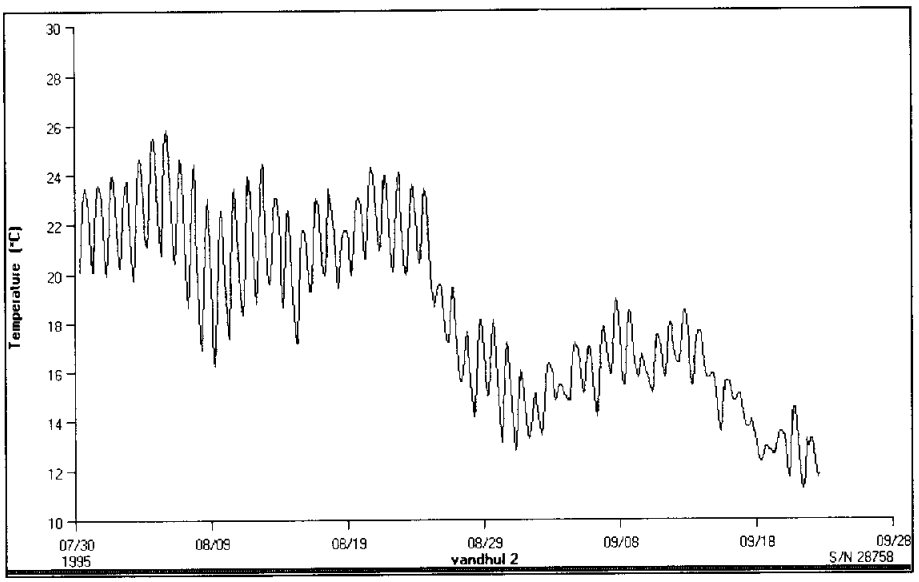


Figure 3.10

Temperature at the bottom during the experimental period in pond 2. The pattern of the temperature in pond 3, 4 and 5 are not different from that of pond 2.

Temperaturforløb på bunden i undersøgelsesperioden for vandhul 2. Temperaturforløbet i vandhul 3, 4 og 5 er ikke forskelligt fra forløbet i vandhul 2.

Table 3.5

Ions in sediment and water in the ponds. Si:Silicon (water); Na: Sodium; Ca: Calcium; K: Potassium; Cd: Cadmium.

Ioner i sediment og vandfase i vandhuller. Si: silicium (vand); Na: natrium, Ca: kalcium; K: kalium; Cd: kadmium.

Ions Unit	Si mg/l	Na mg/kg	Ca mg/kg	K mg/kg	Cd mg/kg
Pond 2	5,16	13,02	46,94	0,842	0,338
Pond 3	6,97	14,97	72,87	1,1	0,465
Pond 4	9,9	18,13	51,09	0,349	0,379
Pond 5	6,61	17,91	65,54	1,355	0,768

Table 3.6

Results of analysis of samples of surface water (10 cm). Analysed for the active substance esfenvalerate. Results are in ng/l.

Analyseresultater af vandprøver taget 10 cm under overfladen. Der er analyseret for den aktive stof esfenvalerat. Resultaterne er angivet i ng/l.

Date / Nominal conc.	22/8 1½ hour after spraying	23/8 24 hours after spray-ing	24/8 48 hours after spray-ing	13/9 21 days after spraying
Reference (pond 2)	<20	<20	<20	<20
35 ng/l (pond 4)	112	32	<20	<20
77 ng/l (pond 3)	90	30	<20	<20
132 ng/l (pond 5)	187	132	<20 trace	<20

The higher measured concentrations are achieved because the ponds are sprayed at the surface without stirring. This causes a gradient through the water column.
It should be noted that the samples were kept in a freezer for 1½ year before the analysis. It is not known what influence the time delay has on the results.

3.2.2 **The cage experiment**

The cages were placed in the ponds on August 18 1995, five days before spraying.

Almost all animals died.

All animals in the cages except a few replicates in pond 3 died. The explanation for this may be the very low oxygen concentrations in the bottom in periods (see *Figure 3.9*) combined with the high water temperatures (*Figure 3.10*). The animals in the cages had no opportunity to escape the unfavorable conditions.

3.2.3 **Surface activity and behaviour**

Ændret adfærd

In the pesticide treated ponds, the surface activity and behaviour of surface breathing animals, especially Dytiscidae (water beetles) and Corixidae (water boatman) changed notable after spraying (see *Table 3.7)*, whereas no changes were seen in pond 2, the reference pond. 1½ hour after the spraying many animals in the treated ponds were seen on the surface as well as along the shores. The animals did not react to disturbance i.e. did not try to escape. After 24 hours, the animals showed normal behaviour (avoidance reactions and no animals climbing the shores) but the animal activity was generally lower in the ponds treated with the two highest doses compared to the activity in the reference pond.

Table 3.7

Surface activity and behaviour of Dytiscidae and Corixidae. All observation in one minute.

Overfladeaktivitet og adfærd af Dytiscidae og Corixidae. Alle observationer er foretaget i 1 minut.

Pond (nominal conc.) / date	Pond 2 reference	Pond 4 35 ng/l	Pond 3 77 ng/l	Pond 5 132 ng/l
22/8 1½ time after spraying	6 animals breathing at the surface. No other activity. Reacts by escaping at disturbance.	Approx. 35 animals on surface. Large activity. Crawling on the shore. Do not react at disturbance.	15 - 20 animals at surface. Large activity in surface and at bottom. Crawling on the shore. Do not react at disturbance.	Approx. 50 animals in surface. Few active, many immobile, some on the shore. No reaction to disturbance.
23/8 24 after spraying	5 breathing on the surface. Normal behaviour.	10 breathing on the surface. Dead animals drifting on the surface. Otherwise normal behaviour.	2 breathing on the surface. Dead animals drifting on the surface. Otherwise normal behaviour.	3 breathing on the surface. Dead animals drifting on the surface. Otherwise normal behaviour.
24/8 48 after spraying	10 breathing at surface. Normal behaviour.	7 breathing at surface. Normal behaviour.	2 breathing at surface. Normal behaviour.	2 breathing at surface. Normal behaviour.

3.2.4 **Benthic invertebrates**

The densities of animals collected by plastic tubes are shown in *Table 3.9, Table 3.10, Table 3.11* and *Table 3.12.* Sampling has been carried out three times within a month around the spraying time August 22 1995, and a additional sampling was carried out 8 month later to detect possible long-term effect.
Because of a procedural mistake in sorting, some samples have been lost before identification of species. Therefore, only higher taxa are included.

Electro-fishing

After the experiment was started, fish were seen in pond 3. October 16 1995 the ponds were Electro-fished with the following result. No fish were caught in ponds 1, 2 and 4, whereas perch (Perca fluviatilis) were found in pond 3 (eighty specimens, 5-6cm), pond 5 (nine specimens, 8-10cm, and pond 6 (seven specimens).

Thus, pond 3 had an overcrowded community of perch. The perch were no doubt introduced to the ponds as eggs or larvae together with the implanted sediment and vegetation from the natural pond.

Influence on fauna composition and biomass.

16 months went from the introduction of fish until the Electro-fishing. It seems reasonably to assume that the fish in the three ponds had similar conditions of temperature, water quality and as a start condition - feeding potential. The number of fish caught by Electro-fishing is supposed to reflect the number of fish introduced together with the sediment.

The small perch feed especially on zoo-plankton and benthic invertebrates and they might have had a considerable influence on the fauna composition/density and thereby indirectly on the phytoplankton biomass.

A semi quantitative assessment of the influence of fish on *Asellus* can be made in the following way. An average wet weight of *Asellus aquaticus* is 25 mg (see figure 2.2). The number of *Asellus* per m^2 in the beginning of the esfenvalerate mesocosms experiment appears from table 3.9, 3.10, 3.11 and 3.12. The biomass of Asellus can then be estimated, see *Table 3.8.*

Table. 3.8
Effect of fish predation on Asellus in the esfenvalerate mesocosms experiment (17-08-95, before treatment).

Pond/esfenvalerate ng/l	Pond 2 Reference	Pond 4 35	Pond 3 77	Pond 5 132
Pond area m^2	30	25	21	25
Asellus biomass g/pond	506	338	47	422
number of perches	0	0	80	9

Table 3.8 indicates that in pond 3 the 80 perches had an influence on the number of Asellus while this was not apparent in pond 5. The nine perches in pond 5 did not reduce the number of Asellus up until the start of the esfenvalerate mesocosms experiment.

The winter 95/96 was very cold and the ponds were ice-covered until mid April. The ice cover might have had an influence on the species composition/density of invertebrates in the ponds due to oxygen depletion. The ice was up to 30cm thick but the ponds were never frozen to the bottom at the deepest part that range from 60cm to 80cm.

Table 3.9

Pond 2 (reference pond). Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 2 (reference vandhullet). Aritmetrisk gennemsnit og 95% c.l. af 10 Kajakrør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2

Date	17-08-95		24-08-95		21-09-95		07-05-96	
	<x>	±95% c.l.	<x>	±95% c.l.	<x>	±95% c.l.	<x>	±95% c.l.
Gastropoda	315	349	1890	1129	945	609	855	464
Lammellibranchia	90	118	90	118	90	176	90	118
Hirudinea	180	452	360	452	90	118	90	118
Oligochaeta	1530	1206	2655	1701	1440	1000	10755	7688
Malacostraca	675	378	1935	1360	1215	417	135	135
Ephemeroptera	180	195	315	349	765	645	180	144
Odonata	180	188	225	237	405	158	135	118
Heteroptera	135	188	90	118	135	135	0	
Coleoptera	135	195	0	88	0		0	
Trichoptera	270	195	90	176	360	523	135	135
Nematocera	0		360	288	675	620	1755	760
Sum	3690	1480	8145	2790	6120	1722	14130	9207

Table 3.10

Pond 3 (1/8 doses Sumialpha®) Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 3, 1/8 dosis Sumialpha®. Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2.

Taxa/Date	17-08-95		24-08-95		21-09-95		07-05-96	
	<x>	±95% c.l	<x>	±95% c.l	<x>	±95% c.l	<x>	±95% c.l
Gastropoda	2205	1165	1350	696	1800	683	1575	1256
Lammellibranchia	1170	994	675	781	1050	725	450	348
Hirudinea	180	144	180	269	150	93	45	88
Oligochaeta	315	456	450	294	2450	1409	9360	6446
Malacostraca	90	118	0		50	93	0	
Ephemeroptera	0		0		0		45	88
Odonata	135	188	45	88	0		90	118
Heteroptera	0		0		0		0	
Coleoptera	0		0		0		0	
Trichoptera	90	118	45	93	0		45	88
Nematocera	90	176	270	88	500	380	1215	647
Sum	4365	1950	3015	1250	6000	2254	12825	7919

Table 3.11

Pond 4 (1/16 doses Sumialpha®) Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 4, 1/16 dosis Sumialpha®. Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2 .

Taxa/Date	17-08-95		24-08-95		21-09-95		07-05-96	
	<x>	±95% c.l	<x>	±95% c.l.	<x>	±95% c.l.	<x>	±95% c.l
Gastropoda	720	399	855	425	585	421	800	479
Lammellibranchia	135	188	45	88	315	363	300	197
Hirudinea	45	88	225	237	180	93	100	93
Oligochaeta	315	295	630	545	1035	1072	9500	4637
Malacostraca	540	585	135	188	405	279	0	
Ephemeroptera	180	195	0		45	93	50	93
Odonata	45	88	225	147	270	186	300	197
Heteroptera	45	88	0		135	139	0	
Coleoptera	135	271	0		0		0	
Trichoptera	0		0		0		0	
Nematocera	180	144	675	479	315	335	1350	776
Sum	2340	870	2790	1000	3285	1293	12400	6763

Tabel 3.12

Pond 5 (¼ doses Sumialpha®) Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 5, ¼ dosis Sumialpha®. Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2 .

Taxa/Date	17-08-95		24-08-95		21-09-95		07-05-96	
	<x>	±95% c.l	<x>	±95% c.l	<x>	±95% c.l	<x>	±95% c.l
Gastropoda	1935	1616	810	390	1440	718	450	372
Lammellibranchia	135	135	45	88	450	372	135	135
Hirudinea	0		0		45	88	45	88
Oligochaeta	630	300	630	959	810	555	3465	5536
Malacostraca	675	421	540	614	0		0	
Ephemeroptera	270	269	0		0		0	
Odonata	45	88	180	195	90	118	0	
Heteroptera	0		45	88	45	88	0	
Coleoptera	45	88	45	88	0		0	
Trichoptera	0		45	88	0		0	
Nematocera	315	265	45	88	360	317	855	867
Sum	4095	1830	2385	1190	3240	1222	4950	5458

3.2.5 **Analysis of results**

Please refer to chapter 7, addendum: Statistical Analysis of Mesocosms Experiment by Per Homann Jespersen. Statistical considerations and methods are explained and all statistical results are shown fully.

In the diagrams shown in the following and the diagrams in addendum, the unit of the ordinate are least squares means estimates of mean. The diagrams are identical and statistical conclusions are based upon the addendum. Ephemeroptera were at first excluded from statistical analysis because of the reasons mentioned in addendum 1, but the species collected in this investigation are small and not rapidly mobile, why the sampling strategy may not be very imprecise regarding mobility. The sampling strategy is designed for benthic invertebrates. Ephemeroptera, in this case *Cloeon dipterum,* are a substrate animal, feeding on the substratum, but more pelagic than true benthic animals.

In all figures, pond 3 (CONC$ B) are shown only for explorative reasons, but are excluded in any discussion or consideration of results, because of the influence of fish.

Explanation of the following diagrams:
The following diagrams shows the least squares means as a function of respectively concentration (CONC$) and sampling dates (DATE$). 0: Reference pond, A: lowest concentration (pond 4), B: MEDIUM concentration (pond 3) and C: Highest concentration (pond 5). Unit for the dependent variable (ordinate), is number of animals per subsample (= per plastic tube sample). Points marked with S.E. (standard error).

Figurer visende mindste kvadraters hovedvirkning som funktion af hhv. koncentration (CONC$) og prøvedatoer. 0: referencevandhul, A: mindste koncentration (vandhul 4), B: mellemste koncentration (vandhul 3) og C: højeste koncentration (vandhul 5). Enheden for den afhængige variabel, Y-aksen, er antal dyr pr. subsampel (= pr. kajakrør prøve). Punkter med S.E. (standard error).

May flies and midge

- **Ephemeroptera, Nematocera and Trichoptera:** The only insects included in the statistical analysis. There is a significant difference for Ephemeroptera (*figure 3.11)* between 0 and the other levels. A dose-response effect might be indicated. These results indicates a nearly exclusion of Ephemeroptera because of the pesticide. There may be a similar acute effect on all insects as seen in the behavioural observations for surface animals, with the exception that inwater juveniles have no possibility to escape. Ephemeroptera were not able to re-colonize the ponds up to May 96.

  Nematocera shows re-colonisation (*figure 3.12)* and a dose response effect might be indicated.

The statistical analysis for Trichoptera gives significant differences between the reference pond and pond 4 and 5, but the data for Trichoptera should not be not included. The effect can not be ascribed to the application of pesticide but rather to the very low number of specimen in pond 4 and 5.

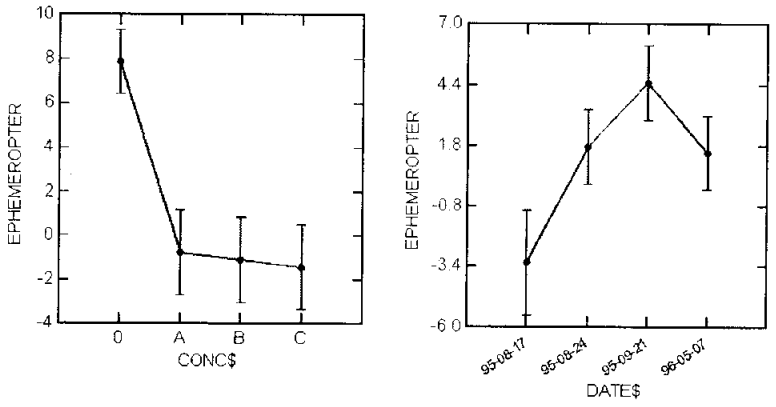


Figure 3.11

Ephemeroptera. May flies.

Ephemeroptera, døgnfluer.

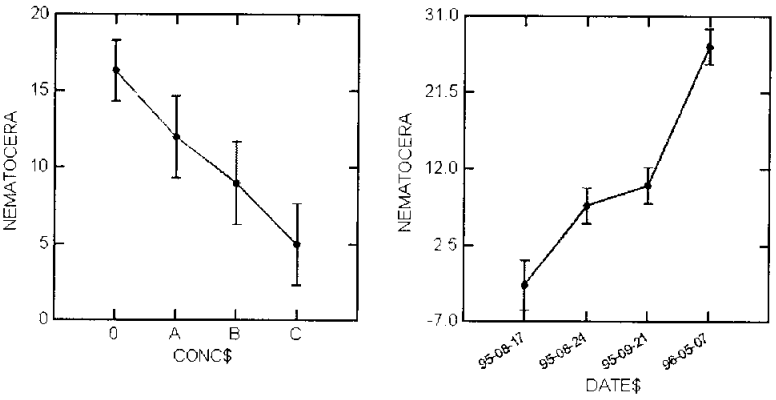


Figure 3.12

Nematocera (midge).

Nematocera (myg).

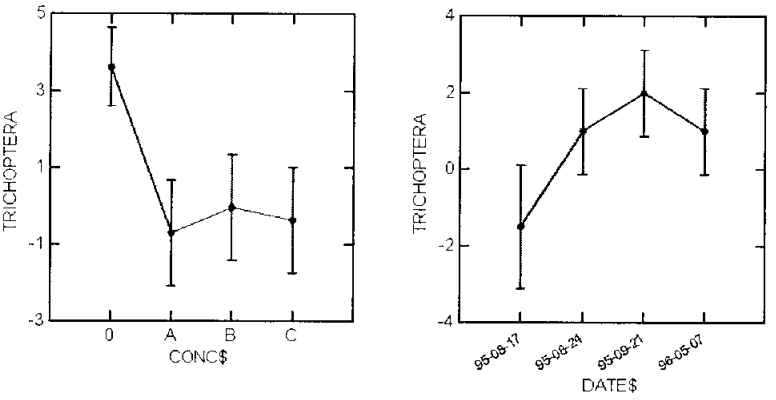


Figure 3.13

Trichoptera (caddis fly)

Vårfluer

Crustacean, freshwater isopods.

- **Malacostraca (*Asellus aquaticus*):** There is a significant difference between the reference pond and the sprayed ponds. As the sampling strategy are designed for the sampling of benthic animals and especially for the sampling of Asellus the difference between the reference pond and the others should be regarded valid. The substratum at the sampling area in the reference pond and the sampling area in the other mesocosms are identical. There are no significant differences between dates.

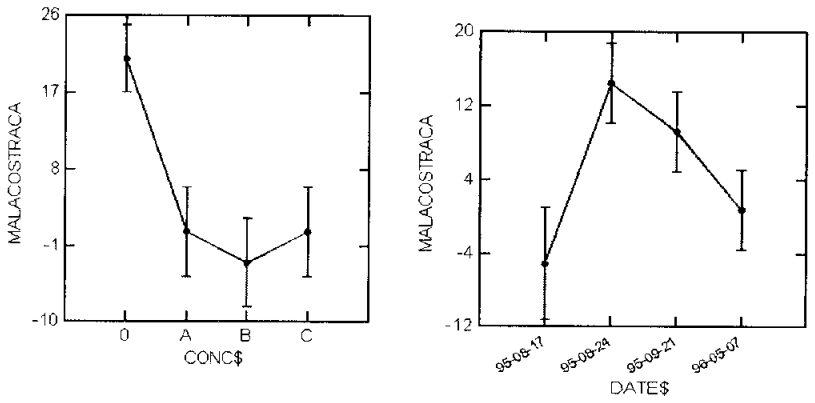


Figure 3.14

Malacostraca (Crustaceans, here only Asellus aquaticus
Malacostraca (Storkrebs, her kun Asellus aquaticus

Snails and mussels

- **Gastropoda and Lammellibranchia:** Gastropoda (snails) (*figure 3.15*) and Lammellibranchia (mussels) (*figure 3.16*) exhibit the same pattern for concentration and date dependency. There is no significant difference in between concentrations or between different dates. Especially for Gastropoda, there is a tendency for aggregation, but confidence limits are high, therefore interpretation is difficult. Any conclusion should be taken with care, but seems that neither of the groups is affected by the pesticide.

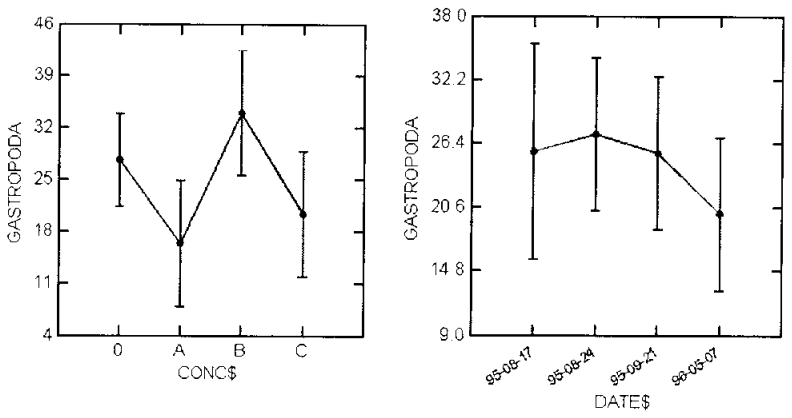


Figure 3.15

Gastropoda.

Gastropoda.

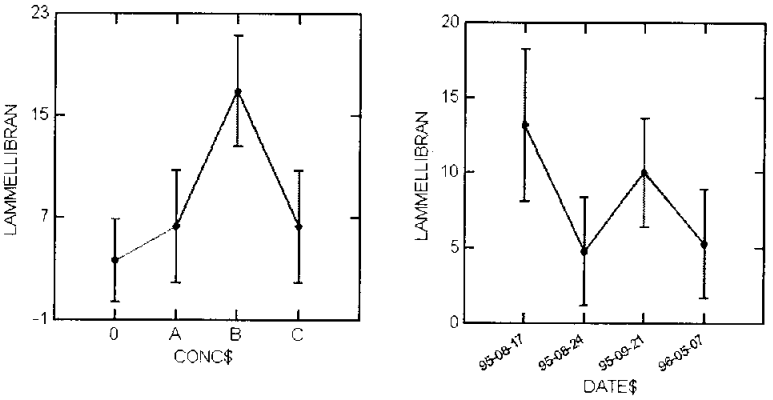


Figure 3.16

Lammellibranchia.

Lammellibranchia.

Bristle worms

- **Oligochaeta:** Oligochaeta (bristle worms) have no significant concentration dependency when using turkey adjustment, but a significant difference between reference pond and pond 5 and reference pond and pond 4 and 5. So there might or might not be a dose respond. Interpretation should be taken with care, because of a tendency to aggregation of animals. There is a significant increase in density in the long-term experiment until 7/5-95, but not in the short-term experiment.

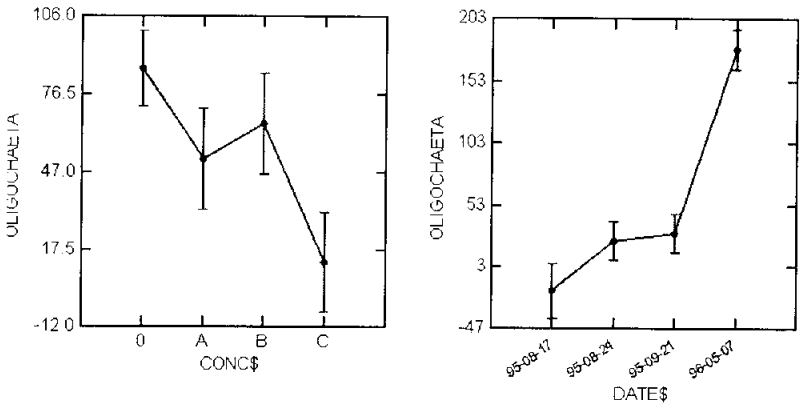


Figure 3.17

Oligochaeta.

Oligochaeta.

3.2.6 **Periphyton**

The results of the algae experiment are presented in *Figure 3.18*. Each point is an average of 5 replicates with 95% C.L.. In the beginning the periphytic algae biomass in pond 2 (reference) was significant higher than the biomass in the treated ponds. In the end of the experiment the biomass in the reference pond was significant lower compared to the treated ponds. There was no relation between esfenvalerate concentration and the final biomass levels. The final biomass of pond 3 and 4 were not significant different and was significant higher then the final biomass in pond 5.

Algae experiment. Each point is an average of five replicates and showing 95% C.L.

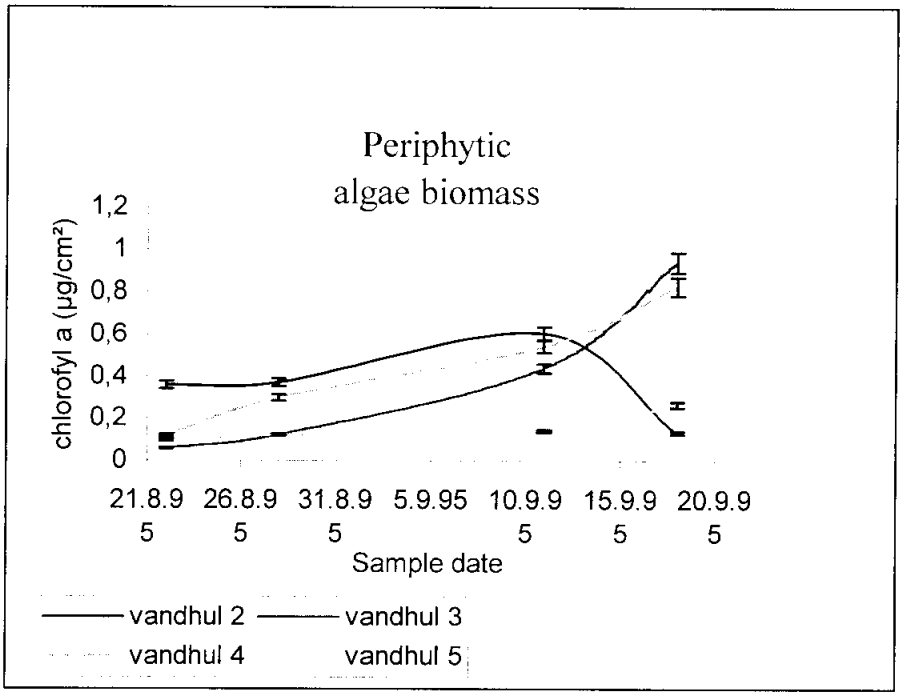


Figure 3.18

Algeforsøg. Hvert punkt er et gennemsnit af 5 replikater med 95% c.l..

3.3 Discussion of experiments with Sumialpha® and esfenvalerate

EC50 - values

References of EC50 values for *Asellus* and esfenvalerate have not been found. The DEPA authorisation (Miljøstyrelsen, 1988) includes an acute toxicity of $LC50^{48h}$ of 48ng/l for *Daphnia pulex*. This value is between the $EC50^{48h}$ values found in the present study for *Asellus* and Sumialpha® with and without organic material. In a review, Day (1989) found that LC50 values for *Daphnia magna* were in the same magnitude as LC50 values found for other crustacean. Fairchild et al. (1992) found $LC50^{48h}$ of *Daphnia magna* to be 270ng/l (esfenvalerate). In a test, Baughman et al (1989) found with fenvalerate and the brackish water shrimp *Palaemonetes pugio* values between 13 and 52ng/l.

The EC50 values of *Asellus* in the present study are therefore considered as similar level as other known values. The values are presumably underestimated, because pyrethroids are lipophilic chemicals that are known to adsorb to the glass in bioassays (Sharom and Soloman, 1981). When the concentrations were given as nominal instead of analysed concentrations Day and Kaushik (1987) found that use of glass containers underestimated the toxic effect with one third. Fairchild et al. (1992) found that the presence of sediment reduced the bio availability threefold. In the present study, a difference of 40 times is found between $EC50^{48h}$ with and without organic material (Figure 3.1 and Figure 3.2). The difference may be explained by the enlarged surface due to alder leaves in the aquarium.

Against that background, it is reasonable to assume that a concentration of 100ng/l esfenvalerate will affect *Asellus* and other crustaceans in a natural environment depending on the amount of and surface of sediment and organic material. I found a variation in the $EC50^{48h}$ from 2.5 to 110ng/l esfenvalerate. The tests were performed at 10°C in 1 l aquaria with 2.5ng/l esfenvalerate nominal in water without organic material and 110ng/l esfenvalerate nominal in water with organic material.

As is true for this experiment as for others, it not possible to evaluate the mode of effect, that is whether the effect are caused by directly contact or indirectly by feeding organic matters with adsorbed pesticide. However, the effects are not purely qualitative for that reason, because the effects are measurable and correlated by a factor of a nominal concentration.

Effects on growth

A significant inhibition of the growth is observed from concentrations six times lower than the $EC50^{48h}$ with organic material. Esfenvalerate in the trade product formulation Sumialpha® has the largest effect. The temperature has a considerable influence on the growth (Andersson, 1969 and Økland, 1979) as does the size of the animal, as the growth rate decreases with bigger size (Sutcliffe et al., 1981). Furthermore, the quality of the food influences the growth rate (Willoughby and Marcus, 1979; Welton and Clarke, 1980; Pedersen, 1990). Consequently it is difficult to compare growth rates from the present report with other studies, since parameters such as food, temperature and animal size seldom are alike. As an example, Willoughby and Marcus (1979) found, for *Asellus aquaticus*, a specific growth rate of 4.85 and 2.24%/day (wet weight) at 15°C at an initial length of 2.5mm, feeding on respectively conditioned oak leaves and Streptomyces (actinomycet). The corresponding growth rates of animals with an initial

length of 1mm were 6.81 and 2.74%/day. In the present report the initial length for the smallest size group in the two growth experiments in the control group is 6.2mm and 5.3mm, respectively. The corresponding specific growth rates (wet weight) are 1.4%/day for both groups. The two size groups are so close that the difference has no influence on the growth rate (wet weight), but the growth rate (wet weight) is much less than was found for corresponding food items by Willoughby and Marcus (1979). The temperature and the initial length were different.

For the present study, the experimental conditions were equal, and the results from the growth experiments can be analysed statistically with the concentration as the only variable parameter.

As mentioned earlier, it is true for this experiment as for others that it is not possible to evaluate the mode of effect, in other words whether the effect is caused by direct contact, or indirect contact via feeding organic matters with adsorbed pesticide. However, the effects are not purely qualitative for that reason, because the effects are measurable and correlated by a factor of a nominal concentration.

Ecotoxicological tests should include the formulated trade product, not only the technical pure pesticide.

Sumialpha® had a larger growth inhibition at sublethal concentrations than technical pure esfenvalerate. The reason for this might be the auxiliary chemicals added to Sumialpha®. Therefore, the relevance of the existing practice, only testing the active substance should be considered. According to Peter Leeuwangh, DLO, Winand Centre for Integrated Land, Soil and Water Research, Wageningen (personal communication, May 1995) it is most relevant to use formulated pesticides in ecotoxicological research projects. Formulated pesticides are used in toxicity tests from this laboratory (Brook et al., 1992; Van Donk et al., 1995; Brock et al., 1995, Cuppen et al., 1995). The present study supports this opinion. The inhibition of the growth rate (length) of size group small *Asellus aquaticus* was approximately 64% for 18.75ng/l and 100% for 37.75ng/l, by use of formulated esfenvalerate (Sumialpha®), while no inhibition was observed by similar concentrations of technical pure esfenvalerate.

Esfenvalerate in sediment and water.

Between 24 hours and 48 hours after spraying with Sumialpha® the active substance esfenvalerate was dissipated or under the detection limit (20ng/l) in the surface water (10cm) for all the ponds. Fairchild et al. (1992) measured in a mesocosms study a half-life time of 10.4 ±2.0 hour for esfenvalerate. Using that results in the present study the detection limit should be reached within 48 hours. In a littoral enclosure study with esfenvalerate, Heinis and Knuth (1991) found that 90% disappeared from the water phase within 24 hours and reached the detection limit (47ng/l) within 4 days. After 8 days 84% of the esfenvalerate was adsorbed to macrophytes (*Chara* sp.). After 4 days, approximately 18% was adsorbed in the sediment. After 60 days and a repeated spraying of esfenvalerate, 82% was adsorbed in the sediment. On an average 83% of the adsorbed esfenvalerate was present in the upper 1cm of the sediment. The distribution was characterised by two stages. 1) During the first two days most of the esfenvalerate was present in the water phase; 2) after four days, most was adsorbed to macrophytes and the sediment.

In the present study, the measured levels of esfenvalerate in the water phase fit with the referred studies, and it could be assumed that esfenvalerate after 2 to 4 days was adsorbed to macrophytes and the sediment. Benthic shredders such as *Asellus aquaticus* and grazers of filamentous algae such as *Cloeon dipterum* (Brown, 1960) are likely to consume the adsorbed esfenvalerate.

Behaviour of surface-macroinvertebrates.

Changes of behaviour and surface activity were observed 1 1/2 hours after spraying in the treated ponds compared to the reference pond. The increased activity in the surface was dose-response related. In a laboratory experiment, Andersson (1982 and 1989) found that fenvalerate and permethrin could change the behaviour and be lethal to different invertebrates with respective concentrations of 22 and 30ng/l. Three categories of sensitivity were observed for fenvalerate. Amphipods were most sensitive with a loss of co-ordinated muscle control and following death. Different insect groups were next in a range of sensitivity. Snails showed no sign of response. In the present mesocosms study water beetles showed amplified activity and shore climbing behaviour that made them an easy target for terrestrial carnivores.

Aggregation of animals

The findings from the sampling method show that there has been an aggregation of animals. In the reference pond, the sampling showed 315 gastropods on August 17 1.995 while 1.890 gastropods were found 7 days later. Such an increase in number might reflect an aggregation since a reproduction of approximately 1500 snails in 7 days is impossible. The confidence limits are generally wide because of few sub-samples and consequently the interpretations of results are statistically uncertain.

Effects on macroinvertebrates.

Esfenvalerate reduced the number of larvae/nymphs of insects and *Asellus aquaticus* at the lowest concentration (nominal 38ng/l). Snails and bivalves did not show sensibility, but the results are uncertain because of tendency to aggregation. Oligochaeta did not show sensibility with tukey adjustments, and the group increased in number during the long-term experiment. Andersson (1982) found that snails were resistant to fenvalerate even when exposed to concentrations of 790ng/l. In a mesocosms experiment with esfenvalerate Fairchild et al. (1992) found that the total number of Insecta and Gastropoda was reduced by the lowest concentration (250ng/l) applied by the experiment. Diptera and Gastropoda were the most sensible groups, but this was probably due to a failure in the method of collection in his experiment.

Periphyton and indirect effects.

The acute toxicity of esfenvalerate to algae was not examined in the DEPA authorisation (1988) but esfenvalerate should not be directly toxic to phytoplankton for the applied concentrations (Fairchild et al., 1992). Therefore, an effect on periphyton should not be expected. On the contrary, an increase of periphyton biomass during the experiment in the treated ponds occurred and there was a large decline in biomass in the reference pond at the end of the experiment. The increase of biomass in the treated ponds might have been caused by a removal of grazers. The same conclusion but with a significant dose-response increase was found in study of the density of diatoms (Schroll et al., 1998). The snail density showed no significant change but there was a significant decline of the density of Mayflies (*Cloeon dipterum*) in the treated ponds. *Cloeon dipterum* grazes periphytic filamentous algae (Brown, 1960). The increase of periphytic biomass can be interpreted as an

indirect effect although there is not a clear dose-response relation. The predominant decline in biomass in the reference pond can be seen as a response to an increased density of May flies (*Cloeon dipterum*) in the same period.

Influence of fish

The overcrowded community of perch in pond 3 influenced the composition of macroinvertebrates. The higher density of Lammellibranchia and Gastropoda, as far as the data may be regarded as valid, can be explained as a function of less competition of food items and less macroinvertebrate predation of the larvae. As mentioned small perch feed on zooplankton and benthic invertebrates in particular. Both groups are primary consumers and partly competitors to Lammellibranchia and Gastropoda.

Method of sampling.

The experiment has showed a conflict between sampling method and the need of making as little disturbance as possible. The sampling method is designed for benthic invertebrates and is not very suitable for mobile pelagic animals. Furthermore, the number of subsamples and the number of sampling events make conclusions about unequal distributed animals uncertain. This conflict seems to be very difficult to cope with when making experiments in small mesocosms.

4 The prochloraz experiments - results and discussion

4.1 Laboratory experiments

Growth experiments were carried out with prochloraz in the trade product formulation Sportak 45 EC, which contains 450g/l active substance (prochloraz). Experiments with technical pure prochloraz were not included. The formulated trade product Sportak 45 EC form the actual environmental impact and are therefore ecotoxicologically more interesting, and consequently Sportak 45 EC was used in the experiment.

4.1.1 Sublethal concentrations

$EC50^{48h}$ was estimated for small and medium size *Asellus aquaticus*. No organic materials were added. The effect was immobility or death.

$EC50^{48h}$ was estimated to an average of 665µg/l for small *Asellus aquaticus* and medium *Asellus aquaticus*, nominal concentration (*Figure 4.1*).

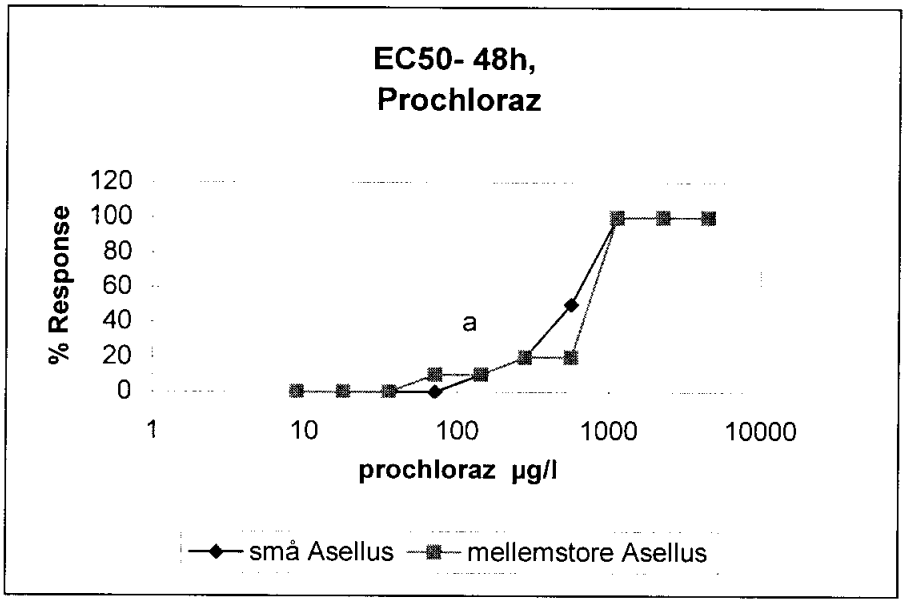


Figure 4.1

EC50 experiment using Asellus aquaticus and prochloraz in the trade formulation Sportak®. $EC50^{48h}$ are estimated to an average of 665µg/l for small and medium size Asellus (nominal). Response: Immobility or death.

EC50 forsøg med Asellus aquaticus og prochloraz tilsat i handelsformuleringen Sportak®. $EC50^{48h}$ er estimeret til et gennemsnit på 665 µg/l for små Asellus og mellemstore Asellus (nominelt). Effekt: Immobilitet eller død.

4.1.2 **Growth experiment with Sportak®**

The growth experiment was accomplished in the laboratory air-conditioning plant in 34 days at 15°C. 500 *Asellus aquaticus* were applied in three size groups with 10 animals in each 1 l aquarium and 3-5 replica in each size group and concentration. The concentrations appear from *table 4.1.* All the concentrations were nominal concentrations of prochloraz. Prochloraz was added as the trade product formulation Sportak®. The food was supplied as alder leaves (5 leaves 630.23 ±29.45mg, 95% C.L., n=96).

Table 4.1

Results from growth experiments with Asellus aquaticus (L) in different concentrations of prochloraz. Small Asellus from hole size 1-1.5mm; medium Asellus 1.5 – 2.0mm; big Asellus 2.0 – 2.5mm. n=500.

Resultater fra vækstforsøg med Asellus aquaticus (L) i forskellige koncentrationer af prochloraz i formuleringen Sportak®. Små Asellus udsorteret fra hulstørrelse 1 - 1,5mm; mellem Asellus : 1,5 - 2,0mm; store Asellus 2,0 - 2,5mm. n=500.

TREATMENT/ ANIMAL SIZE	Length (mm) ± 95% C.L.	WW (mg) ± 95% C.L.	DW (mg) ± 95% C.L.
SMALL			
initial	5.1 ±0.2	5.07 ±0.50	0.75 ±0.09
control	5.3 ±0.2	5.43 ±0.70	1.22 ±0.14
70 µg/l	5.2 ±0.2	5.30 ±0.78	1.28 ±0.15
140 µg/l	5.2 ±0.2	5.41 ±0.74	1.23 ±0.15
280 µg/l	5.4 ±0.2	5.76 ±0.72	1.26 ±0.14
560 µg/l	5.1 ±0.3	5.57±1.03	1.12 ±0.20
MEDIUM			
initial	6.6 ±0.2	10.27 ±0.74	1.42 ±0.16
control	6.5 ±0.2	10.35 ±0.76	2.28 ±0.27
70 µg/l	6.6 ±0.2	10.58 ±0.85	2.08 ±0.29
140 µg/l	6.7 ±0.2	10.94 ±0.79	2.45 ±0.28
280 µg/l	6.8 ±0.1	10.73 ±0.82	2.55 ±0.28
560 µg/l	6.5 ±0.2	9.80 ±0.95	2.17 ±0.33
BIG			
initial	7.7 ±0.2	15.34 ±0.97	2.53 ±0.19
control	8.0 ±0.2	17.13 ±1.24	3.33 ±0.29
70 µg/l	7.7 ±0.3	16.01 ±1.17	3.24 ±0.29
280 µg/l	7.9 ±0.3	17.32 ±1.37	3.50 ±0.33
1120µg/l	7.7 ±0.5	16.87 ±2.39	3.09 ±0.55

Small animals: Initial end holes size 1.5 mm. Growth experiment 34 days.
Medium animals: Initial end holes size 2.0 mm. Growth experiment 34 days.
Big animals: Initial end holes size 2.5 mm. Growth experiment 34 days.

No replicates were omitted. All the animals grew significantly in relation to the initial dry weight (DW) ($p<0.00$) but not in length and wet weight (WW) ($0.11<p<0.67$, ANOVA, multiple range analysis). There is no significant difference between control parameters and parameters of the different concentrations for all three size groups ($0.15<p<0.61$, ANOVA, multiple range analysis).

Mortality in the experiment

Small and medium size *Asellus aquaticus* had a mortality at 560µg/l that was significant higher than for lower concentrations including the control ($p<0.05$, ANOVA, multiple range analysis). For big *Asellus aquaticus* the mortality was significant highest at 1120µg/l., subsequently at 280µg/l, which was significant higher than lower concentrations and the control (ANOVA, multiple range analysis). See *table 4.2.*

Table 4.2

*Mortality in % and 95% C.L. in the growth experiment with Asellus aquaticus and Sportak® (see table 4.1). Small: 5 replicates; medium: 4 replicates; big: 3 replicates. n=500. Small: Mortality at 560µg/l is significantly higher than other concentrations (ANOVA, multiple range analysis, P<0.04). Medium: Mortality at 560µg/l is significantly higher than other concentrations (ANOVA, multiple range analysis, P<0.05). Big: Mortality at 1120µg/l significantly higher than mortality at 280µg/l, which are significantly higher than mortality at 70µg/l and control (ANOVA multiple range analysis, P<0.003). *=Significance.*

Mortalitet i % med 95% C.L. under Asellus aquaticus vækstforsøg med prochloraz formuleret som Sportak® (se table 4.1) Små: 5 replikater, mellem: 4 replikater, store: 3 replikater, n=570. Små: Mortaliteten ved 560 µg/l er signifikant højere end de øvrige koncentrationer (ANOVA multiple range analysis, P<0.04). Mellem: Mortaliteten ved 560 µg/l er signifikant højere end de øvrige koncentrationer (ANOVA multiple range analysis P<0.05). Store: Mortaliteten ved 1120 µg/l er signifikant højere end mortaliteten ved 280 µg/l der igen er signifikant højere end mortaliteten ved kontrollen og 70 µg/l (ANOVA multiple range analysis P<0.003). .= signifikans*

	Control	70µg/l	140µg/l	280µg/l	560µg/l	1120µg/l
Small	15 ±21	32 ±21	30 ±21	26 ±21	64 ±21*	
Medium	18 ±12	25 ±12	20 ±12	28 ±12	43 ±12*	
Big	33 ±17	17 ±17		47 ±17*		73 ±17*

Specific growth rate

Growth rate in dry weight (DW) was calculated as the specific growth rate Gs (% per day).

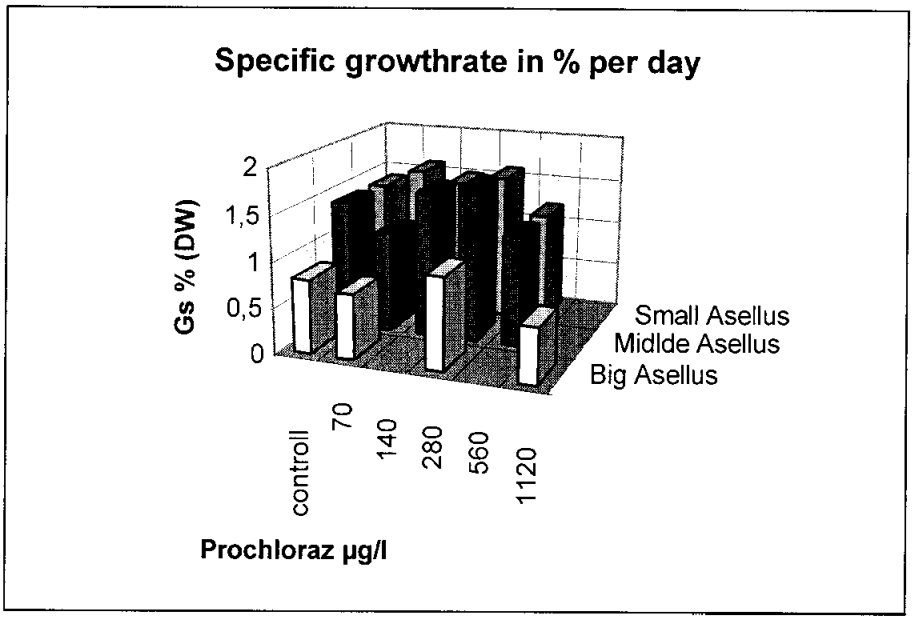
$$Gs = (\ln(\text{end parameter (value)} / \text{initial parameter (value)})) * t^{-1}) * 100\ \%$$

(Sutcliffe et al. 1981).

The specific growth rates for dry weight (DW) are shown in *figure 4.2.*

Figure 4.2

Specific growth rate (dry weight) in % per day. n=720 animals. There is no significant difference in Gs between control and other concentrations, irrespectively of sizes. Big animals have significant lower Gs than medium and small animals.



From *table 4.1* and *figure 4.2* it appears that Sportak® did not show a significant effect on the growth rate for the chosen concentrations. Big animals had a lower growth rate than small and medium size animals.

4.1.3 Summary

EC50^{48h}

No significant differences were seen in EC50^{48h} for small and medium size animals. The average EC50^{48h} is estimated to be 665µg/l (nominal).

Growth rate in Sportak® experiment

There was no significant effect of Sportak® on the growth rate for small, medium or big *Asellus aquaticus* for the chosen concentrations.

Mortality

Sportak® had a significant effect on the mortality of *Asellus aquaticus* at 560µg/l (nominal) for small and medium size animals. For small animals the mortality increased from an average of 25% to 64% and for medium size animals from an average of 23% to 43%. For the big animals, the mortality increased from an average of 25% to 47% at 280µg/l (nominal) and up to 73% at 1120µg/l (nominal). These values correspond well with the EC50^{48h} values. Apparently, addition of organic material had no effect.

4.2 Pond experiments (mesocosmos)

Results from the pond experiments are presented in the following. The experiments were started May 7 1996 and finished June 6 1996. There was no possibility to assess the long-term effect of prochloraz (Sportak®).

The prochloraz mesocosms experiment was carried out with the ponds already used for the esfenvalerate experiment. The reference pond was the same, and prochloraz concentrations were introduced in random selected ponds. The reuse of ponds was not optimal but the resources were not sufficient to empty and refill the ponds. Although the ponds had a recovery pe-

riod of approximately nine months an overlapping effect from the esfenvalerate experiment can not be excluded. As mentioned earlier fish were removed from pond 3 and 5 by Electro-fishing October 16, 1995. No fish were observed since then. It is very possible that an influence still exists on the fauna composition although the effects should be minor compared to the influence on the experiment of esfenvalerate, taking the recovery period into consideration.

4.2.1 Chemical data the sampling area

pH

The average pH in the ponds was 8.77 ±0.25 (95% C.L.) with a standard deviation of 7% and a maximum, minimum of 9.99 and 7.89 (*Figure 4.3*).

Oxygen

The surface oxygen concentration was on an average 12.6mg/l (8-18.3) over the period (*Figure 4.4*) while the bottom oxygen on an average was 10.0mg/l (1-16.5) and lowest in ponds 3 and 4 (*Figure 4.5*).

Temperature

The temperature varied in the beginning of the period until May 18.1996 with an average of 10°C (7°C - 15°C), and afterwards the temperature increases in a regular manner from 8°C to approximately 17°C. (*Figure 4.6).*

Ions

The analysed ions in the water and the sediment appear in *Table 4.3.*

Sportak®

The analyses of water samples after spraying with Sportak® are presented in *Table 4.4.* The active substance prochloraz was analysed by DTI.

Pond 2 was the reference. Pond 4 was sprayed twice normal dose, pond 3 with normal dose, and pond 5 received half of normal dose.

Detectable concentrations of prochloraz were still found in the surface water after 16 days.

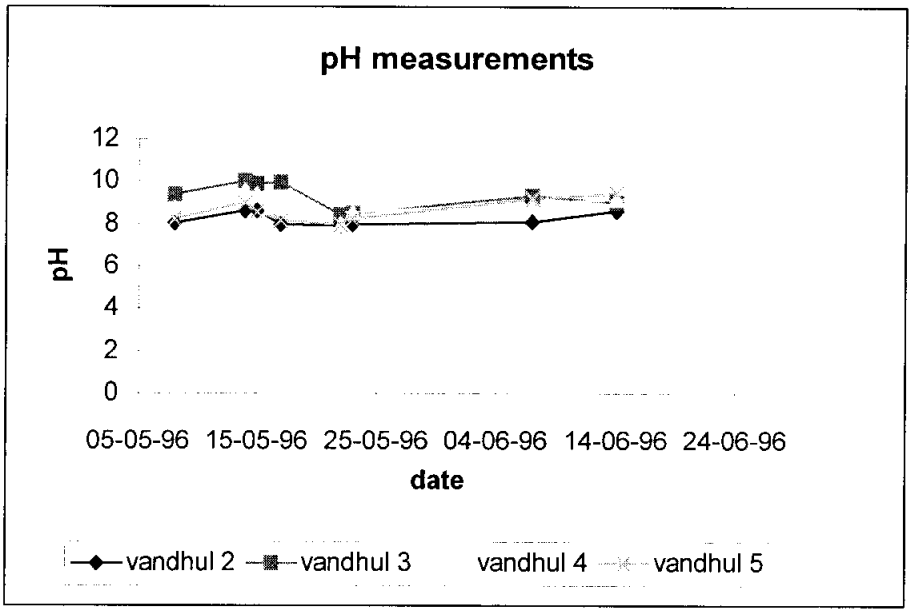


Figure 4.3

pH-measurements. The average pH is 8,77 ±0,25 (95% C.L.).

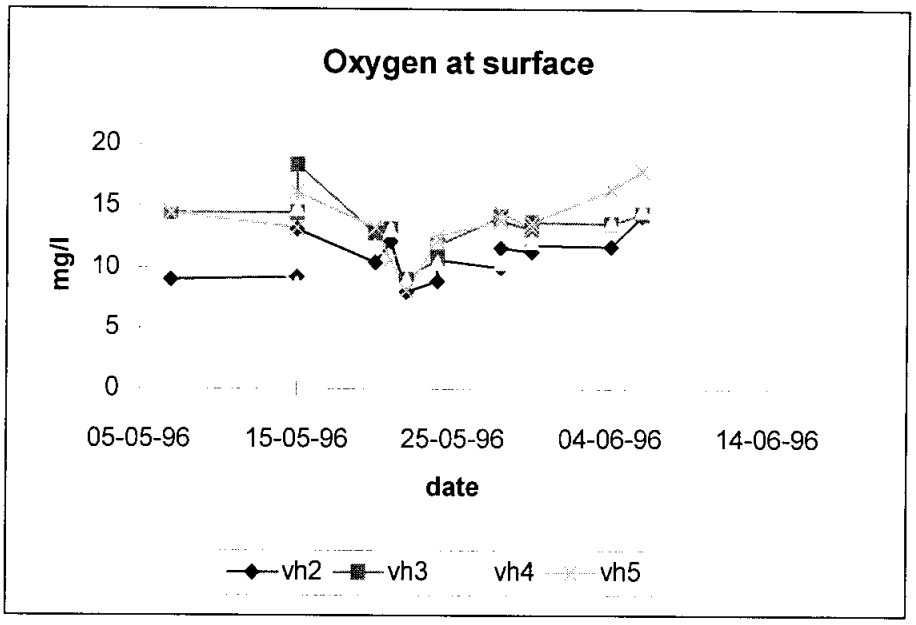


Figure 4.4

Oxygen in mg/l, 10 cm beneath surface

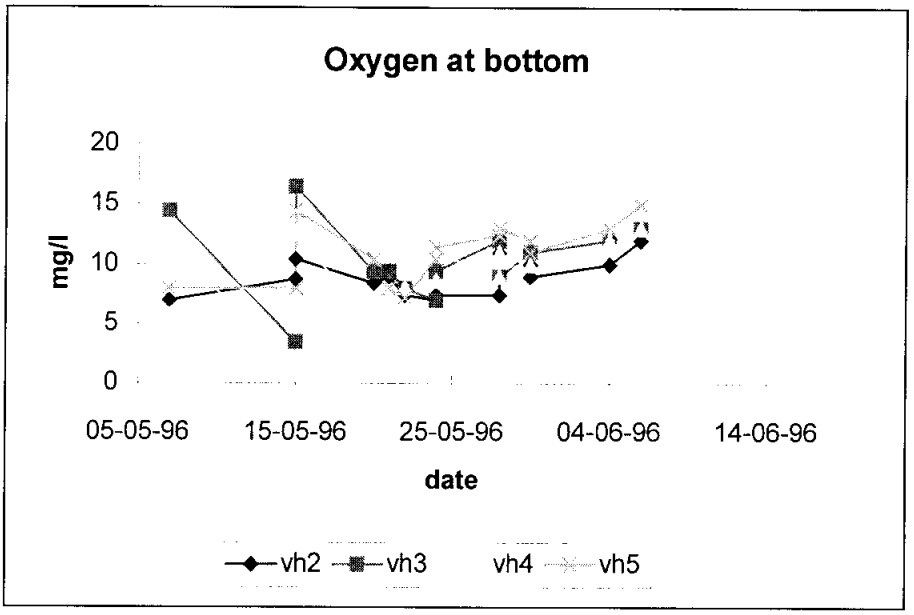


Figure 4.5

Oxygen measured at the bottom. In mg/l.

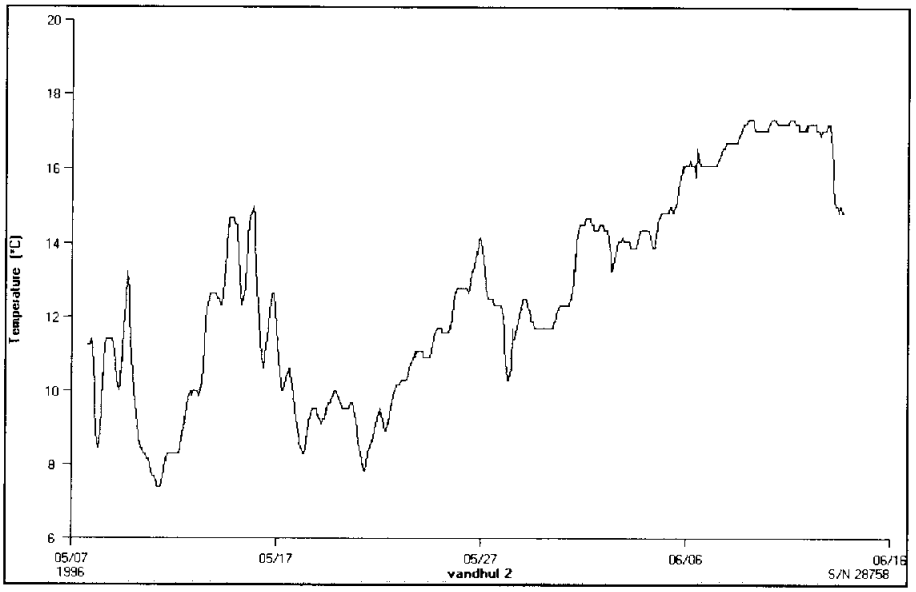


Figure 4.6

The temperature progress on the bottom in pond 2 in the experimental period. The temperature progress in the other experimental ponds is not different.

Table 4.3

Ions in sedimet and water in the ponds. Si: Silicon; Na: Sodium; Ca: Calcium; K: Potassium; Cd: Cadmium (sediment).

Ioner i sediment og vandfase i vandhuller. Si: silicium; Na: natrium, Ca: kalcium; K: kalium; Cd: cadmium (sediment).

	ions/ unit	Na mg/l	K mg/l	Ca mg/l	Si mg/l	Cd mg/kg
Pond	May 7	0,43	<0,05	2,16	1,21	0,28
2	June 4	0,43	<0,05	2,11	0,52	
Pond	May 7	0,37	<0,05	1,17	0,71	0,45
3	June 4	0,41	<0,05	1,26	1,58	
Pond	May 7	0,48	<0,05	1,24	0,33	0,51
4	June 4	0,53	<0,05	1,25	0,57	
Pond	May 7	0,54	0,52	1,74	0,45	2,62
5	June 4	0,55	0,05	1,32	0,61	

Table 4.4

Results of water samples taken 10 cm. beneath surface. Analysed for the active pesticide Prochloraz. Results in µg/l.
Analyseresultater af vandprøver taget 10 cm. under vandoverfladen. Der er analyseret for det aktive stof prochloraz. Resultaterne er angivet i µg/l.

Pond/ Date	13/5 before spraying	13/5 after spraying	14/5	23/5	29/5
Pond 2	<0,02	<0,02	<0,02 trace	0,2	<0,02 trace
Pond 5	<0,02	30	35	2	1
Pond 3	<0,02	106	74	10	6
Pond 4	<0,02	155	150	22	14

4.2.2 **Cage experiment**

The cages were placed May 9 1996 with 3 replicates and 8 *Asellus aquaticus* from size group small in each. Alder leaves were added as food. The cages were collected May 29 1996.

Growth parameters and specific growth rate (Gs)

In *Table 4.5* is the growth parameters shown. All the animals have grown significantly compared to the initial parameters (ANOVA, multiple range analysis, $p=0$), but there was no significant difference between the growth in the treated ponds and the reference pond (ANOVA, multiple range analysis, $0.62<p<0.85$). The specific growth rate is shown in *Figure 4.7* calculated as Gs in % per day after Sutcliffe et al. (1981).

Table 4.5

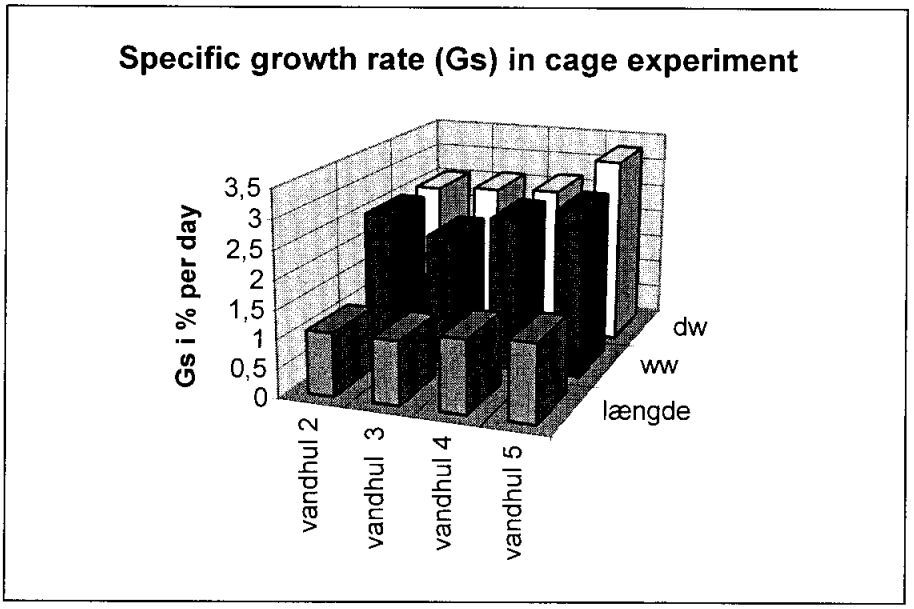
Results from cage experiments with Asellus aquaticus, placed in the mesocosms. Small Asellus aquaticus were used, form hole sizes of sorting apparatus 1 –1.5mm. n=121

Resultater fra burforsøg med *Asellus aquaticus* udsat i forsøgsvandhullerne. Der er brugt små Asellus udsorteret fra hulstørrelsen 1-1,5 mm. n=121. Forsøget varede 20 dage. Tal i ( ) er fraktion af normal dosis.

Pond	Length (mm) ± 95% C.L.	WW (mg) ± 95% C.L.	DW (mg) ± 95% C.L
initial	4,9 ±0,3	5,29 ±1,25	1,09 ±0,30
pond 2 (ref.)	6,1 ±0,2	8,78 ±1,36	1,81 ±0,32
pond 4 (2/1)	6,3 ±0,6	8,97 ±2,28	1,85 ±0,56
pond 3 (1/1)	6,1 ±0,5	8,27 ±1,61	1,83 ±0,40
pond 5 (1/2)	6,4 ±0,4	9,40 ±1,55	2,10 ±0,37

Figure 4.7

Specific growth rate in % per day for small Asellus in cages put out in mesocosms. No significant difference in growth rate between the reference pond and the treated ponds.



Mortality

The mortality in the cage experiment appears from *Table 4.6*. The trend of a dose-response relation is not significant (ANOVA, multiple range analysis, p=0.47).

Table 4.6

Mortality in cage experiment with Asellus aquaticus placed in mesocosms. Mortality in % (minimum, maximum).

Mortaliteten i burforsøg med Asellus aquaticus udsat i forsøgsvandhullerne. Mortaliteten er vist i % med minimum, maksimum i parentes.

Pond	Pond 2 ref.	Pond 5 ½ dose	Pond 3 1/1 dose	Pond 4 2/1 dose
mortality %	29 (25-37)	43 (13-68)	50 (13-100)	75 (25-100)

4.2.3 **Surface activity and behaviour**

The surface activity and behaviour of surface breathing animals, especially Dytiscidae (water beetles) and Corixidae were observed for 1 minute. In pond 2, the reference pond, nothing changed in the period. No changes in surface activity or behaviour were observed after spraying. Dead animals were found floating on the surface of the treated ponds 2 hours after spraying.

Table 4.7

Surface activity of Dytiscidae and Corixinae. All observations are carried out in 1 minute.

Overflade aktivitet af Dytiscidae og Corixinae. Alle observationer er foretaget i 1 minut.

Pond / date	Pond 2 reference	Pond 4 2/1 dose	Pond 3 1/1 dose	Pond 5 ½ dose
13/5 before spraying	2 animals breathing at surface. Escaping behaviour.	4 animals breathing at surface. Escaping behaviour.	1 animal breathing at surface. Escaping behaviour.	3 animals breathing at surface. Escaping behaviour.
13/5 2 hours after spraying	3 animals breathing at surface. Escaping behaviour.	1 animal breathing at surface. Escaping behaviour. Dead animals at surface.	2 animals breathing at surface. Escaping behaviour. Dead animals at surface	3 animals breathing at surface. Escaping behaviour. Dead animals at surface
14/5 24 hours after spraying	3 animals breathing at surface. Escaping behaviour.	2 animals breathing at surface. Escaping behaviour. No dead animals at surface.	2 animals breathing at surface. Escaping behaviour. No dead animals at surface.	3 animals breathing at surface. Escaping behaviour. No dead animals at surface.

4.2.4 **Benthic invertebrates**

The densities of animals collected by plastic tubes are shown in *Table 4.8, Table 4.9, Table 4.10* and *Table 4.11.* Sampling has been carried out three times: 6 days before spraying with Sportak® (May 7 1996), 7 days after spraying (May 21 1996) and 23 days after spraying (June 6 1996)

The winter 95/96 was very cold and the ponds were ice-covered until the middle of April. This might have had an influence of the density of inverte-

brates in the ponds. The ice was up to 30cm thick and the ponds were never frozen to the bottom on the deepest part that range from 60cm to 80cm.

Table 4.8

Pond 2 (reference pond). Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 2 (reference vandhullet). Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2 .

Taxa/Date	07-05-96		21-05-96		06-06-96	
	<x>	±95% c.l	<x>	±95% c.l.	<x>	±95%c.l.
Gastropoda	855	464	225	147	405	425
Lammellibranchia	90	118	0		0	
Hirudinea	90	118	0		0	
Oligochaeta	10755	7688	12555	8520	7425	4123
Malacostraca	135	135	0		180	353
Ephemeroptera	180	144	180	269	135	188
Odonata	135	118	90	88	90	88
Heteroptera	0		0		0	
Coleoptera	0		0		0	
Trichoptera	135	135	135	188	45	88
Nematocera	1755	760	1260	811	450	277
Sum	14130	9207	14445	10000	8730	539

Table 4.9

Pond 3 (1/1 dose of Sportak®). Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 3, 1 dosis Sportak®. Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2.

Taxa/Date	07-05-96		21-05-96		06-06-96	
	<x>	±95% c.l.	<x>	±95%c.l	<x>	±95%c.l
Gastropoda	1575	1256	1395	1005	945	565
Lammellibranchia	450	348	405	277	585	695
Hirudinea	45	88	90	118	90	118
Oligochaeta	9360	6446	9225	8016	7380	4447
Malacostraca	0		0		45	118
Ephemeroptera	45	88	0		0	
Odonata	90	118	0		0	
Heteroptera	0		0		0	
Coleoptera	0		0		0	
Trichoptera	45	88	0		90	118
Nematocera	1215	647	765	396	135	188
Sum	12825	7919	11880	8560	9270	5550

Table 4.10

Pond 4 (2/1 dose of Sportak®). Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 4, 2 gange dosis Sportak®. Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2.

Taxa/Date		07-05-96		21-05-96		06-06-96
	<x>	±95% c.l.	<x>	±95% c.l.	<x>	±95% c.l.
Gastropoda	800	479	1300	1057	855	382
Lammellibranchia	300	197	400	325	720	686
Hirudinea	100	93	100	123	0	
Oligochaeta	9500	4637	3650	1466	2655	2101
Malacostraca	50		50	95	0	
Ephemeroptera	50	93	150	197	0	
Odonata	300	197	150	93	45	88
Heteroptera	0		0		45	88
Coleoptera	0		0		0	
Trichoptera	0		50	93	45	88
Nematocera	1350	776	50	95	180	144
Sum	12450	6763	5900	2289	4545	2420

Table 4.11

Pond 5 (½ dose of Sportak®). Arithmetical average and 95% C.L. of 10 plastic tube collections each sampling date. Number of animals per m^2.

Vandhul 5, ½ dosis Sportak®. Aritmetrisk gennemsnit og 95% c.l. af 10 Kajak-rør indsamlinger for hver indsamlingsdato. Antal dyr pr. m^2.

Taxa/Date		07-05-96		21-05-96		06-06-96
	<x>	±95% c.l.	<x>	±95% c.l.	<x>	±95% c.l.
Gastropoda	450	372	945	445	1170	576
Lammellibranchia	135	135	180	235	540	317
Hirudinea	45	88	0		90	118
Oligochaeta	3465	5536	13500	12465	10755	4633
Malacostraca	0		0		0	
Ephemeroptera	0		45	88	0	
Odonata	0		45	88	0	
Heteroptera	0		0		0	
Coleoptera	0		0		0	
Trichoptera	0		0		0	
Nematocera	855	867	810	343	360	342
Sum	4950	5458	15525	12990	12915	7020

4.2.5 **Analysis of the results**

Please refer to chapter 7, addendum: Statistical Analysis of Mesocosms Experiment by Per Homann Jespersen. Statistical considerations and methods are explained and all statistical results are fully shown.

In the diagrams shown in the following and the diagrams in appendix the unit of the ordinate are least squares means estimate of main. The diagrams are identical and statistical conclusions are based upon the addendum.

Explanation of the following diagrams:
The following diagrams are showing the least squares means as a function of respectively concentration (CONC$) and sampling dates (DATE$). 0: Reference pond, A: Lowest concentration (pond 5), B: Medium concentration (pond 3) and C: Highest concentration (pond 4). Points marked with S.E. (standard error).

Figurer visende mindste kvadraters hovedvirkning som funktion af hhv. koncentration (CONC$) og prøvedatoer. 0: referencevandhul, A: mindste koncentration (vandhul 5), B: mellemste koncentration (vandhul 3) og C: højeste koncentration (vandhul 4). Punkter med S.E. (standard error).

May flies and midge

- **Ephemeroptera, Nematocera and Trichoptera:** The only insects included in the statistical analysis. The order Trichoptera,and Ephemeroptera were only sparsely present in the samples and the starting point are very different from the reference pond. Therefore, from an ecological point of view the results are not regarded as valid and left out of consideration. Nematocera (midge, *figure 4.10)* had a significant decrease in the treated ponds compared to the reference pond. There is a significant difference between first and last sampling date.

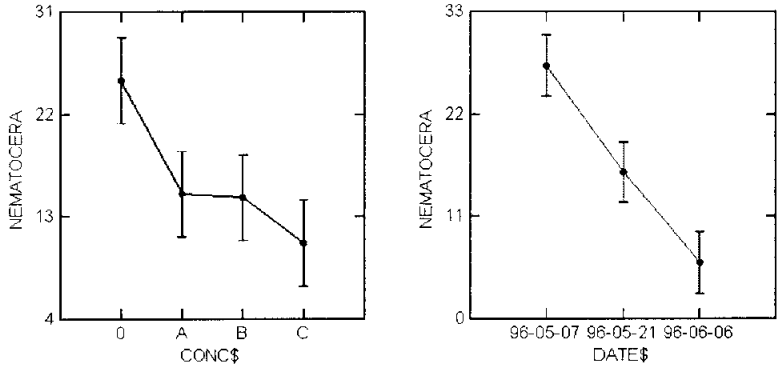


Figure 4.08

Nematocera, Midge.

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Nematocera, myg.

Bristle worms

- **Oligochaeta:** There are no significant effects on Oligochaeta, neither between concentration nor between dates.

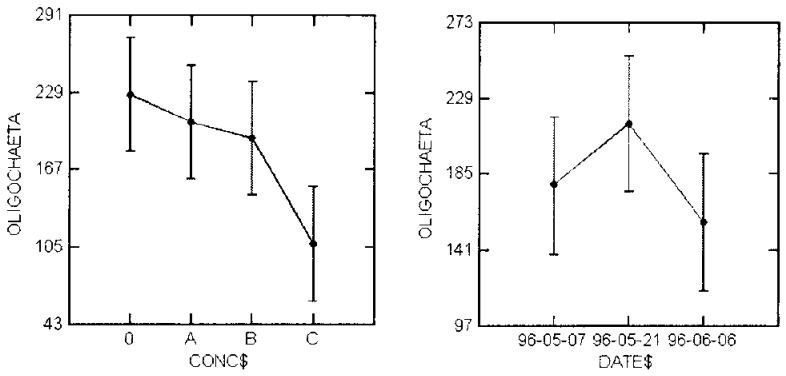

Figure 4.9

Oligochaeta, Bristle worms.

Oligochaeta, børsteorme.

Snails

- **Gastropoda:** Gastropoda (snails) has only a significant increase in density in pond 3. There are no significant differences between dates.

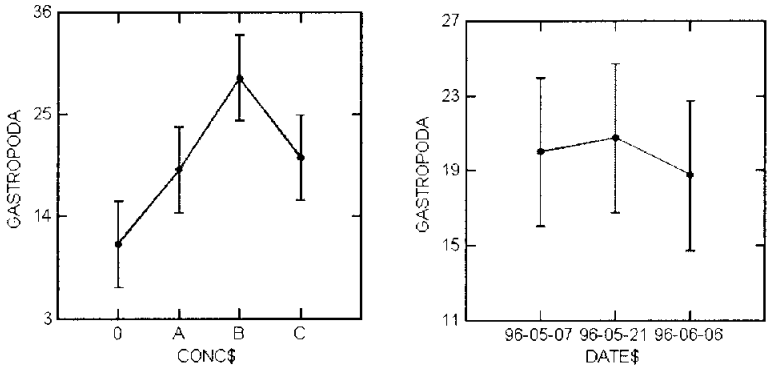

Figure 4.10

Gastropoda (Snails).

Gastropoda.

Crustaceans, leeches and mussels.

- **Malacostraca, Hirudinea and Lammellibranchia:** Are only sparsely present in samples or has an inexplicable disappearance in reference pond. The are therefore left out of consideration.

4.2.6 **Periphyton**

The results of the algae experiment appear from *Figure 4.13*. Each point is an average of 5 replicates with 95% C.L.. In the beginning the periphytic algae biomass in pond 4 was significant higher than the biomass in the other ponds, while the algae biomass in pond 4 was significant lowest on the final date (p=0). Algae biomass in all treated ponds increases significantly. All

the treated ponds have a significant lower algae biomass than the reference pond in the end of the experiment, and pond 4 (the highest prochloraz concentration) has the lowest algae biomass.

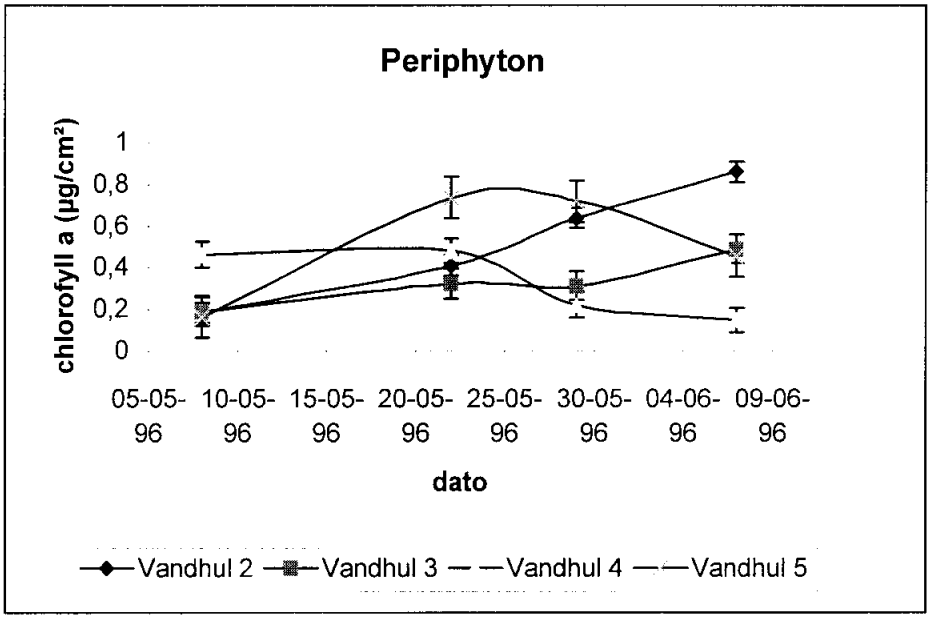

Figure 4.13

Periphyton experiment. Each point is an average of 5 replicates. 95% C.L. is shown.
Algeforsøg. Hvert punkt er et gennemsnit af 5 replikater med 95% c.l..

4.3 Discussion of experiments with Sportak®

EC50 – values.

The average $EC50^{48h}$ for *Asellus aquaticus* is estimated to be 665µg/l (nominal). In an acute toxicity test (Miljøstyrelsen, 1993) with Daphnia magna $LC50^{48h}$ was 2.6mg/l (nominal). In a chronic toxicity test with Daphnia magna in a flow aquarium growth inhibition and reproduction were examined. $LOEC^{21days}$ (lowest observed effect) was 47.2µg/l and $NOEC^{21days}$ (no observed effect) was 22.2µg/l (actual concentrations). In the same experiment, the LC50 was found higher than the tested concentrations. $EC50^{48h}$ for Daphnia was in Tomlin (1995) 4.3mg/l. The estimated EC50 for *Asellus aquaticus* is on this background of the same magnitude or lower than the expected. If it is assumed that LC50 can be appraised from the mortality of the growth test the $LC50^{34days}$ for small to big *Asellus aquaticus* is between 280µg/l to 1120µg/l, nearest to 560µg/l (nominal). This value is in the range of a half to a quarter of the referred values of Daphnia.

Effect on growth

No significant effect on the growth rate was found at the selected concentrations (maximum 1120µg/l nominal). At the highest concentrations a non-significant trend are seen. A growth experiment with higher concentrations and/or tests with single animals may clarify the trend. Apparently, the lethal

concentration LC50 and the effect concentration EC50 are of the same magnitude.

Sportak® in water phase

The chemical analysis showed that 3 to 9% of the initial concentration of prochloraz was present in the upper 10 cm of the water phase 16 days after the spraying. Prochloraz is not easily biodegraded in water (Miljøstyrelsen, 1993), the substance being stable in water at pH7 and 20°C (Kidd and James, 1991), and relative ready soluble in water (55mg/l) (Kidd and James, 1991). A minor decline in oxygen concentration was measured in the period, perhaps due to the degradation of Sportak®. Other factors such as heavy showers in the period could have had an influence.

Behaviour

There was not, as was the case with Sumialpha®, an increased surface activity of the animals. Changes of behaviour such as avoidance to disturbance or fleeing from the water were not observed. Instead, an increased mortality was found.

Cage experiment

As in the laboratory experiment, no significant effect on the growth rate was found. Thus, all the animals grew during the experiment. A non-significant trend towards increased mortality after a dose response relation was found. No other effects were found in the cage test than in the laboratory test. The actual concentrations in the ponds were less than the nominal concentration in the growth experiments in the laboratory, which caused increased mortality. The average higher mortality in the ponds (approximately 50%) could be caused by factors like higher temperature and in periods of low oxygen concentration at the bottom.

The cage experiment could not clarify the validity of data extrapolation from the laboratory experiment to the field, since

- Sportak® did not have an effect on the chosen sublethal parameter either in the laboratory or in the cages
- The increased mortality documented in the laboratory could not be determined in the cages because of the general enhanced mortality and fewer replicates.

Effects on macro invertebrates

The densities of animals were generally low or with very different starting point or had an inexplicable disappearance in reference. Many groups are therefore left out of consideration. Oligochaeta and Gastropoda showed no response, while the density of Nematocera decreased significantly. This decrease may be due to pupation, hatching or flying from the ponds. A decline in density is seen as well in reference pond as in treated ponds, why the decrease in number not easily can be explained by the treatment. The experiment has not clarified whether there is an effect on macroinvertebrates or not.

Periphyton and indirect effects

Prochloraz is considered very toxic for algae (Miljøstyrelsen, 1993). EC50 for *Scenedesmus pannonicus* was 73µg/l (presumably 96 hours). In the mesocosms-experiments a substantial increase of the algae biomass in the reference pond was seen, and a minor increase in ponds 3 and 5, the two lowest test concentrations. In the end of the experiment, these ponds had equal periphyton biomass. Pond 4 with the highest test concentration has a decline in algae biomass and had the lowest algae biomass at the end of the experiment. A dose response related effect over time was observed. As it

seems that the decline can not be explained from grazing animals, it must be assumed that there was a direct effect of Sportak® on the periphyton biomass.

5 Conclusions

The objective of the present project was to provide information about two pesticides concerning

1. Sublethal effects on *Asellus aquaticus* in microcosms (aquaria)
2. To test the extrapolation of sublethal effects on *Asellus aquaticus* from microcosms to mesocosms (artificial ponds)
3. Effects on macroinvertebrates in mesocosms
4. Effects on periphyton in mesocosms
5. Indirect effects in mesocosms.

1. The laboratory experiments show that the trade product Sumialpha® has a sublethal effect on *Asellus aquaticus*, resulting in a reduced growth rate. The effect seems to be higher with the use of the trade product formulation than with the use of technical pure esfenvalerate, which is the active insecticide. Therefore when testing pesticides, it should be recommended that the trade product formulation should be a part of the test procedure.

   The effect is shown for a concentration lower than 9.75ng/l nominal, although the actual concentration possibly is much lower because of adhesion to glass and organic matter. This concentration is below the detection limit of the analysis executed by DTI. Its is not a part of this project to evaluate where the effect are due to the direct exposure or to feeding on contaminated organic matter. However, the pesticides were introduced to the aquarium approx. 24 hours before animals. Its is therefore reasonable to assume, that the major response are due to feeding.

   No inhibition of growth was observed in laboratory experiments for *Asellus aquaticus* with Sportak® in the selected concentrations.

2. Testing an extrapolation of sublethal effects for laboratory tests (microcosms) to ponds (mesocosms) failed. Almost all the test animals died in the exposed cages in the Sumialpha® mesocosms experiment. In the Sportak® experiment, a general higher mortality was found but no sublethal effect.

3. Sumialpha® seems to have an effect on the density of macroinvertebrates at concentrations achieved by a direct spraying of the ponds with doses lower than recommended used on farmland. However, the results are uncertain because of aggregated animals, high confidence limits, fish and reuse of ponds. No conclusions can be drawn from the Sportak® experiment.

   Sumialpha® had an **acute** effect on the behaviour and surface activity of surface animals. The method of observation seems to be very useful, and should be further developed and used in future experiments.

4. Sportak® had a direct toxic effect on periphyton in the treated ponds.

5. The periphyton biomass in the Sumialpha® treated ponds increased, which may be interpreted as an indirect effect due to elimination of grazers.However, the results are very uncertain because of aggregated animals, high confidence limits, fish and reuse of ponds.

Further recommendations and conclusions.

Sumialpha® must be considered highly toxic to aquatic organisms and the pesticide should not be used on areas and under circumstances where a transport to aquatic environments is a potential risk. The long-term effects of sublethal and indirect effects on aquatic ecosystems are not known. It may be recommended to carry out long-term experiments in a large scale with replicate ponds in order to study these effects. It is not possible to draw a conclusion for Sportak® concerning toxicity on animals, although a higher mortality was seen in laboratory experiments. A toxic effect was observed for algae.

A problem not included in the project.

Another problem is related to the common application of pesticide mixtures in modern farming. The mixture of pesticides has potential synergetic effects that could be studied in a mesocosms experiment.

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7 Addendum, by Per Homann Jespersen.

Statistical Analysis of Mesocosmos Experiments

Per Homann Jespersen

The mesocosmos experiments are modelled as two-way analysis of variance-models, with the concentration and day of sampling as independent variables (CONC$ and DATE$, respectively), and with the numbers of *Gastropoda, Lammellibranchia, Hirudinea, Oligochaeta, Malacostraca, Ephemeroptera, Trichoptera,* and *Nematocera* as dependent variables. *Odonata, Heteroptera,* and *Coleoptera* were disregarded for the statistical analysis because of their relative mobility, which in connection with the sampling strategy might give false results.

Other types of modelling were considered utilising the quantitative information in the independent variables in accordance with the recommendations of the OECD[i]. This was, however, not regarded as fruitful for a number of reasons

- there is not sufficient theoretical understanding of the influence of pesticides on the ecosystem to allow for theoretical modelling – it is even questionable whether a monotone dose-response can be assumed
- there is not sufficient data to allow for empirical modelling

Thus, we stick to a 'classical' ANOVA-approach without transformation of the data.[ii]

As each concentration of pesticide is administered to one pond the effect of the concentration level cannot be distinguished from the effect of the pond.

As there is no replication of experiments interaction between CONC$ and DATE$ cannot be tested. This is a major drawback, as we have no reason to believe that there should be no interaction. Thus, results of the statistical analysis have to be interpreted with caution: Tests of effects is not advisable, but if a significant difference is concluded, the conclusion may be accepted.[iii]

Pairwise comparison tests between concentrations are made without adjustment of test level for the number of tests. Significance thus has to be interpreted with care.

Pairwise comparison tests between dates are performed with Tukey adjustment.[iv]

This study, however, also has an explorative aspect. Thus, we will also show figures of ANOVA test results where no significance has been de-

tected. The figures show the least squares estimate of the main effects along the ordinate axis.

7.1 Esfenvalerate

In the esfenvalerate experiment the number of animals were counted just before the addition of esfenvalerate and at three occasions later, 7, 35 and 264 days after, respectively. Esfenvalerate was added in doses of 1/4, 1/8 and 1/16 of normal dose according to the following scheme:

Pond	Dose	CONC$
No 2	0	0
No 3	1/8	B
No 4	1/16	A
No 5	1/4	C

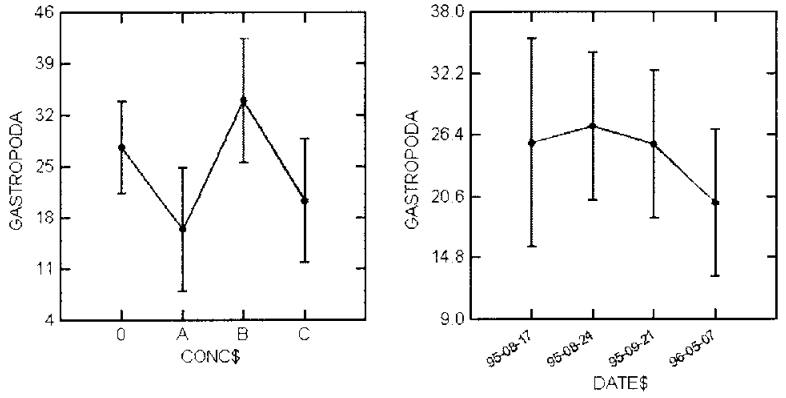
However, in all ponds CONC$ at day zero was set to '0'.

In this experiment perch were found in pond no 3 in a number that probably have influenced the results of the experiment. The presence of a small number of perch in pond no 5 is considered not to have had any notable influence.

In each experiment pairwise comparisons of the number of animals were made between CONC$ 0, A, B, and C. Additionally, the 0-level was compared with the mean of A, B and C (called ABC), and with the mean of A and C (called AC), the latter in order to eliminate the effect of perch in pond no 3. All these comparisons are shown in a table.

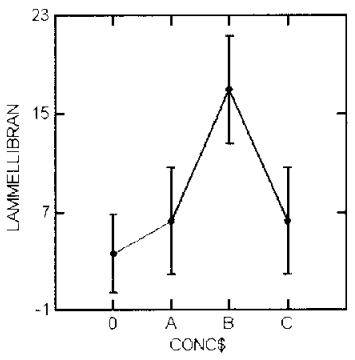
Additionally, pairwise comparisons have been made between the number of animals at the four sampling dates. Tukey adjustment has been applied. Significant differences (p<5%) are reported.

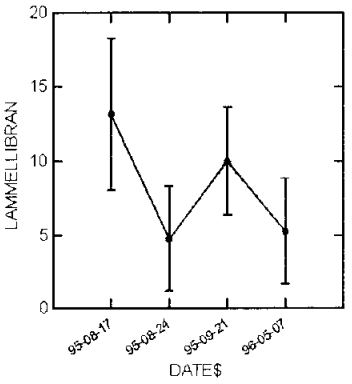
Gastropoda



There are no significant differences, neither between concentrations nor between different dates.

Lammellibranchia



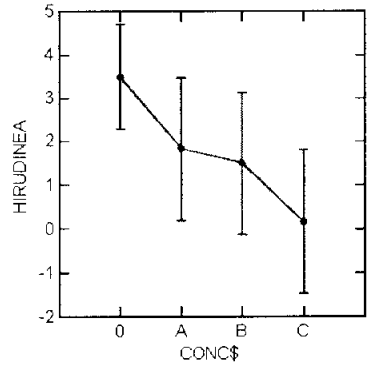


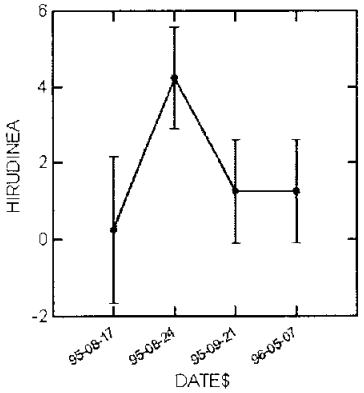
CONC$	A	B	C	ABC	AC
0	>5%	5%	>5%	>5%	>5%
A		>5%	>5%		
B			>5%		

The only significant difference is between the 0 and B-concentrations.

There are no significant differences between dates.

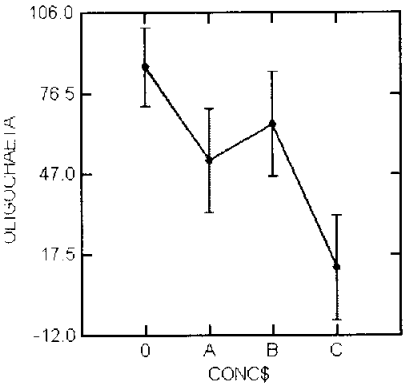
Hirudinea

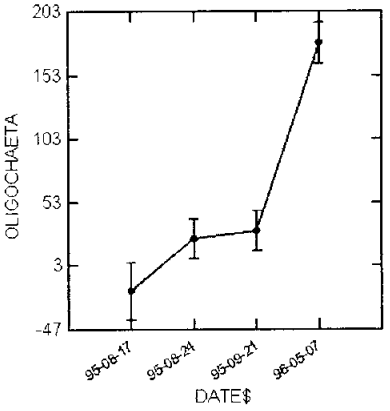




There are no significant differences, neither between concentrations nor between different dates.

Oligochaeta



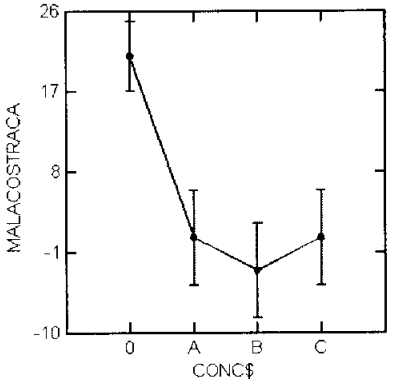


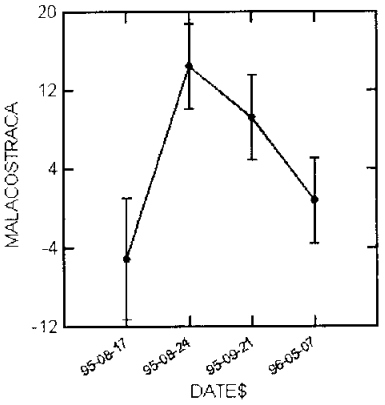
CONC$	A	B	C	ABC	AC
0	>5%	>5%	1,9%	>5%	4%
A		>5%	>5%		
B			>5%		

The 0-level differs significantly from the C- and the AC-level, however not with Tukey adjustment. A dose-response effect might be indicated.

The level at the last sampling date differs significantly from the others.

Malacostraca



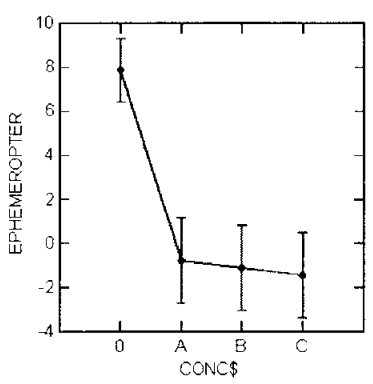


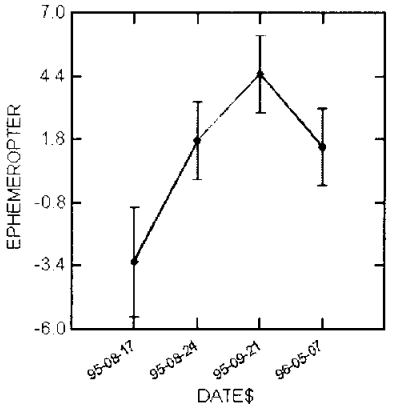
CONC$	A	B	7.1.1.1.1	ABC	AC
0	1,9%	0,8%	1,9%	0,5%	0,9%
A		>5%	>5%		
B			>5%		

A significant difference exists between the 0-level and the other levels.

There are no significant differences between dates.

Ephemeroptera



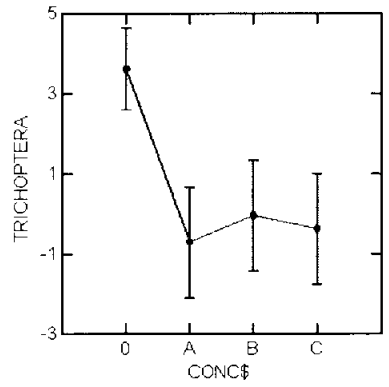


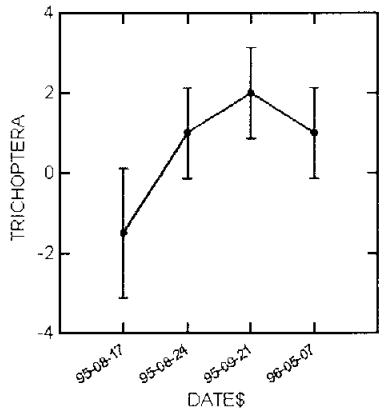
CONC$	A	B	7.1.1.1.2	ABC	AC
0	0,9%	0,7%	0,6%	0,2%	0,3%
A		>5%	>5%		
B			>5%		

A significant difference exists between the 0-level and the other levels. A dose-response effect might be indicated.

There are no significant differences between dates.

Trichoptera



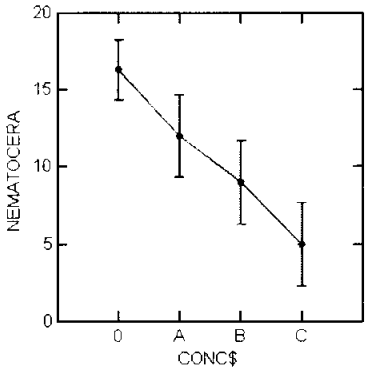


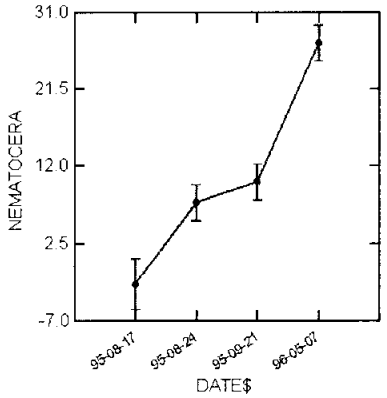
CONC$	A	B	C	ABC	AC
0	4%	>5%	>5%	3%	3%
A		>5%	>5%		
B			>5%		

A significant difference exists between the 0-level and A, ABC and AC-levels.

There are no significant differences between dates.

Nematocera





CONC$	A	B	C	ABC	AC
0	>5%	>5%	1,2%	3%	3%
A		>5%	>5%		
B			>5%		

The 0-level differs significantly from the C-, the ABC- and the AC-levels. A dose-response effect might be indicated.

The level at the last sampling date differs significantly from the others. There is also a significant difference between the first and the third sampling date.

7.2 Prochloraz

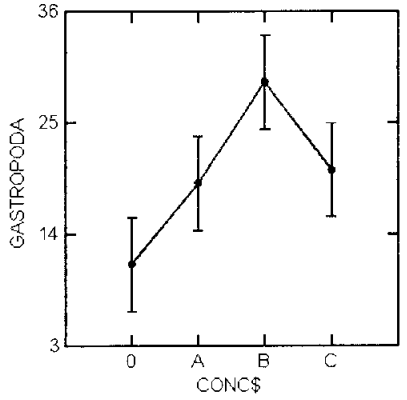
In the prochloraz experiment the number of animals were counted just after the addition of prochloraz and at two occasions later, 14 and 30 days after, respectively. Prochloraz was added in doses of 1/2, 1/1 and two times the normal dose according to the following scheme:

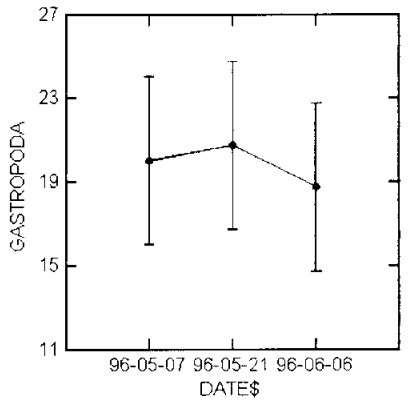
Pond	Dose	CONC$
No 2	0	0
No 3	1/1	B
No 4	2/1	C
No 5	1/2	A

In each experiment pairwise comparisons of the number of animals were made between CONC$ 0, A, B, and C. Additionally, the 0-level was compared with the mean of A, B and C (called ABC). All these comparisons are shown in a table.

Additionally, pairwise comparisons have been made between the number of animals at the three sampling dates. Tukey adjustment has been applied. Significant differences (p<5%) are reported.

Gastropoda



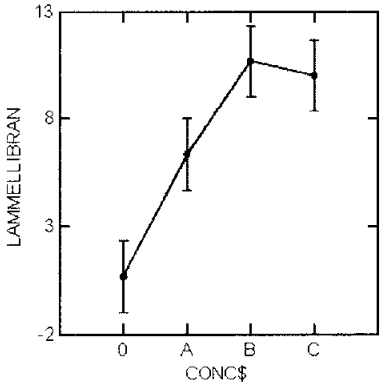


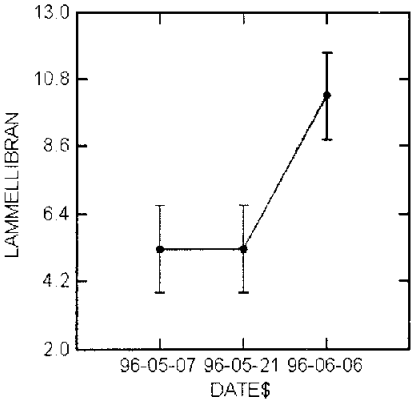
CONC$	A	B	C	ABC
0	>5%	3%	>5%	>5%
A		>5%	>5%	
B			>5%	

The only significant difference is between the 0 and B-concentrations.

There are no significant differences between dates.

Lammellibranchia



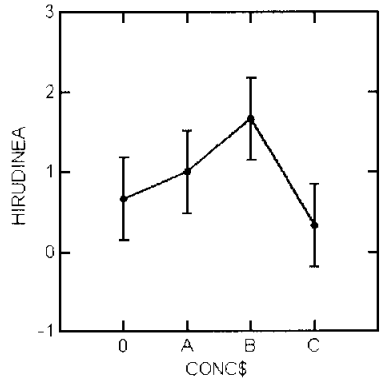


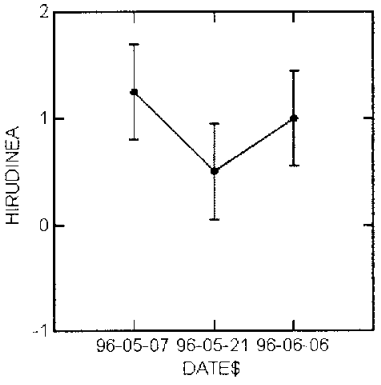
CONC$	A	B	C	ABC
0	>5%	0,5%	0,7%	0,5%
A		>5%	>5%	
B			>5%	

The 0-level differs significantly from the B-, C- and the ABC-levels. A dose-response effect might be indicated.

There are no significant differences between dates.

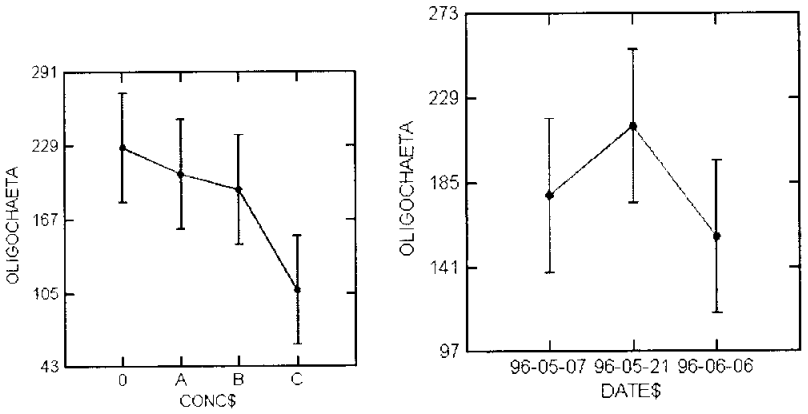
Hirudinea





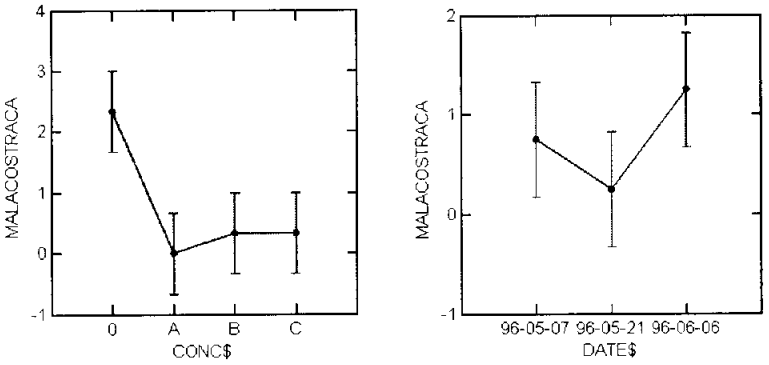
There are no significant differences, neither between concentrations nor between different dates.

Oligochaeta



There are no significant differences, neither between concentrations nor between different dates.

Malacostraca

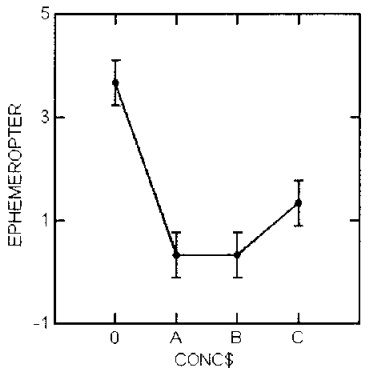


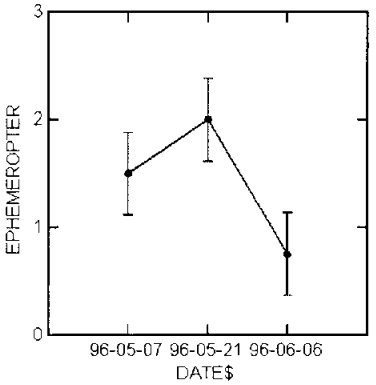
CONC$	A	B	C	ABC
0	5%	>5%	>5%	3%
A		>5%	>5%	
B			>5%	

The 0-level differs significantly from the A- and the ABC-level.

There are no significant differences between dates.

Ephemeroptera



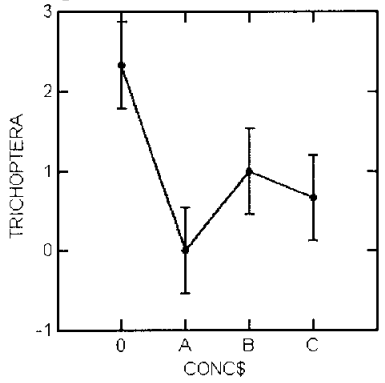


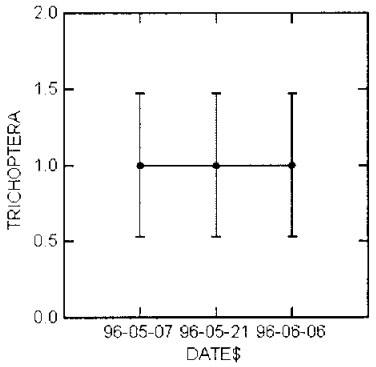
CONC$	A	B	7.2.1.1.1	ABC	AC
0	0,2%	0,2%	1,0%	0,1%	0,2%
A		>5%	>5%		
B			>5%		

A significant difference exists between the 0-level and the other levels. A dose-response effect might be indicated.

There are no significant differences between dates.

Trichoptera



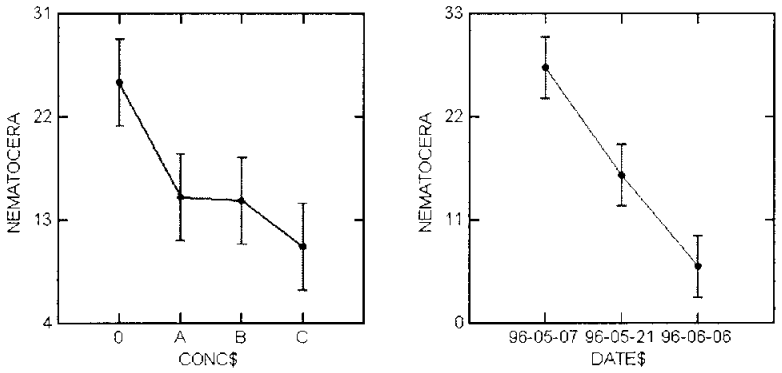


CONC$	A	B	C	ABC
0	2%	>5%	>5%	3%
A		>5%	>5%	
B			>5%	

The 0-level differs significantly from the A- and the ABC-level.

There are no significant differences between dates.

Nematocera



CONC$	A	B	C	ABC
0	>5%	>5%	4%	4%
A		>5%	>5%	
B			>5%	

The 0-level differs significantly from the C- and the ABC-level.

There is a significant difference between the first and the last sampling date.

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[i] *Report of the OECD Workshop on Statistical Analysis of Aquatic Toxicity Data.* OECD Series on Testing and Assessment No. 10, ENV/MC/CHEM(98)18, 1998.

[ii] A square root transformation of the number of animals might give a slightly better accordance with the homogeneity of variance assumption of the variance analysis, but does in preliminary analyses not seem to give very different results. For ease of interpretation the untransformed results are presented.

[iii] J.H. Zar: *Biostatistical Analysis*, 3rd ed., p.252-254. Prentice Hall, 1996.

[iv] When '*there are no significant differences between dates*' is reported, this means that none of the Tukey-adjusted significance levels are less than 5%

8 Appendix

Nominal and measured concentrations of esfenvalerate in laboratory experiments.

Nominal concentration (lab)/ time	Measured concentration (DTI)
9,375 ng/l / 0h	< 20
18,75 ng/l / 0h	< 20 trace
37,5 ng/l / 0h	< 20 trace
75 ng/l / 0h	23
150 ng/l / 0h	35
9,375 ng/l /2 h	< 20
18,75 ng/l /2 h	< 20 trace
37,5 ng/l /2 h	< 20 trace
75 ng/l /2 h	< 20
150 ng/l /2 h	36
9,375 ng/l /24 h	< 20
18,75 ng/l /24 h	< 20 trace
37,5 ng/l /24 h	< 20 trace
75 ng/l /24 h	< 20 trace
150 ng/l /24 h	32
9,375 ng/l /7 days	< 20
18,75 ng/l /7 days	< 20
37,5 ng/l /7 days	< 20 trace
75 ng/l /7 days	< 20 trace
150 ng/l /7 days	< 20 trace

DATA SHEET

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Effects of the Pesticides Esfenvalerate and Prochloraz on Pond Ecology

Subtitle:
With Special Attention on Asellus Aquaticus (L)

Author(s):
Pedersen, Carsten Lauge

Performing organization(s):
Roskilde University. Department of Environment. Technology and Social Studies, Universitetsvej 1, P.O.Box 260, DK- 4000 Roskilde

Abstract:
An insecticide (Sumialpha) and a fungicide (Sportak) are investigated for different effects. Growth inhibitions of Asellus aquaticus were especially elucidated. Growth inhibitions are seen at 9.75 ng/l for Sumialpha. Furthermore, Sumialpha has acute effects on behaviour and surface activity on surface animals in the used test doses.

Terms:
esfenvalerate CAS 66230-04-4; prochloraz CAS 67747-09-5; ecotoxicology; pond ecology; invertebrates

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