Practice Oriented Results on Use and Production of Plant Extracts and Pheromones in Integrated and Biological Pest Control

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BENEFICIALS - THEIR ROLE FOR THE CONTROL OF PESTS INSECTS

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With increasingly intolerable loads of toxic chemicals, including pesticides, for humans and environment scientists and research are called to develop specific biological counteracting procedures and measurements for disturbances and damage, particularly within the area of biological production systems. As defined by Krieg *(1989), the biological plant protection is a "use of organism (antagonist) for the limitation of population of harmful animals and plants" as, for example, the reduction of insect populations by "natural enemies" like entomophags and insect pathogenes.*

1. Use of arthropods

Among arthropods the the number of entomophages are 2 to 3 times as much compared to phytophages, More than 60.000 types of wasp belong to them.

1.1. Predators

Predators means organisms that need more than one prey (beetles and beetle larvae, spirit levels, wasps, ants etc..) for growth and development

1.2. Parasites

Parasites are those entomohages (i.e. wasps) that develop at costs of another animal, either on it (as ectoparasite) or in it (as endoparasite)

2. Use of worms

Nematods are to be diversified as phyto- and zoo-parasitic types, some of them hurt or even kill its insect host by means of associated bacteria specific to the type, which causes Septikaemie (*Heterorhabditis, Steinernema* etc..)

2.1. Nematods

The *Heterorhabditis*-nematods are symbiontic associated with *Xenorhabdus nematophilus*.-bacterium. The bacterial symbiont lives monaxenicly in the intestine of the nematods. Attracted by its insect host (*Galleria mellonella* - wax moth) chemo-tactically the bacteria penetrate by the mouth or percutaneously. The moths are infected by the nematods which, within 24-48 hours, kills the caterpillar. In the cadavers the nematods multiply and after a period of 1-3 weeks a new generation leaves the dead host. For some months these nematods can also survive in the damp soil, even without host contact.

3. Use of microorganisms and infectious agents

The micro-biological plant protection covers the use of fungus, bacteria, viruses, etc.. In insects these pathogenes cause epidemic disease, which leads to the collapse of the population within short time.

3.1. Fungus

Insectopathogenic fungus (mostly from *Fungi imperfecti*, with a by-fruit as conidia (ectosporen)) are penetrated by these conidia under high humidity with a germ hose by integument in the body of insect and developed haemocoel with hyphen and hyphenbody and blastospore. The insect dies due to mycoses by toxin effect or body destruction. The hyphen perforate the cadavers from inside out and form an airmyzel to the cuticula where the characteristic conidia develops. These conidia can persist in upper soil area for a long time. Fungus have a widespread host area - with *Beauveria* and *Metarrhizium*- types, they cover about 200 types of insect and are commercially used for the control of pests in Colorado Potato Beetle *Leptinotarsa decemlineata*, Beetle *Otiorrhynchus sulcatus*, Cockchafer *Melolontha melolontha* etc.)

3.2. Bacteria

The bacteria are the representatives of the Prokaryotes with cells (350-1000 nm) without cell core and without mitotic core phase change. Their genetic material from DNS is localised in a circular "bacterium-chromosome" and in self-reproductive plasmids. The increase of the bacteria takes place via growth and division. The insect pathogenic types infect their host over the digestive tract, after having been taken up with food and are relatively host-specifically. The greatest importance as bio insecticide befits to the Bacillus thuringiensis. Beside the parasporal crystal, which is responsible for the pathotype specific effect on insects, the bacteria trunks even produce 2 exotoxines during their growth in the culture – alfa-exotoxine, a thermo-instable protein, which after injection affects animals non-specifically, and beta-exotoxine, a thermo-stable nucleotide (only with special trunks), which does affect the different insects not only after injection. The commercial bacteria preparations (Dipel, Thuricide HP, Endobakterin, Dendrobazillin etc..) are mostly used against larvae of Lepidoptera in the dose of 300-2000 g (7.5 x 1012 spores + crystals).

3.3 Viruses

Viruses (size less than 250 nm) do not belong to micro organisms, but belong to subcelluar infectious agents and consist of virons, a genetic material of virus nucleic acid in protecting virus protein. The viruses have no material and energy changes and for the biosynthesis of their building blocks they are dependent on alive host cells. The virons do infect the host cells by infiltration or as consequence of the phagocytes. After their biosynthesis the virus components merge to new infectious virons. The replicated viruses are finally removed from the infected cells (budding) or get free by the decay of the host cell (Lyse). Primarily, insectenpathogene viruses

attack in larvae stages after oral accommodation of virons in digestive tract, where the free virons immediately destroy the intestine epithelium and the viruses with containment bodies must be resolved first in the alkaline intestine juice, in order to get the enclosed virons free. With biological plant protection the Baculovirons are particularly used, which occur only with Arthropoden, and which are to be divided into 3 sub-groups:

A – nuclear polyeder viruses against types of leave wasp (*Diprion hercyniae*, *Neodiprion sertifer* (Hym., Diprionidae) in the dose of 4 x 10^7 Polyedern/ha or against *Lymantria dispar* (Lep., Lymantriidae) (10^9) and *Orgyia pseudotsugata* (2.5 10^{12}),etc.

B - Granulose viruses, particularly against *Cydia pomonella* (Lep., Tortricidae) (5x 10^{13}), *Pieris rapae* (Lep., Pieridae),

C - Baculoviron without containment bodies, which strike fat bodies, with very small durability (only 8 days) outside their host - against Indian Beetle *Oryctes rhinoceros*, wax moth *Galleria mellonella*, mosquitos *Aedes vexans* etc..

4. Use of genetic procedures

Is based on the principles of genetic modifications as well as variation and selection in harming exciter/host system.

4.1. Resistance

Acquisition of hosts against harming organisms, including the culture breeding of resistant sorts against harming exciter (Phylloxera vitifoliae, Phytophthora infestans, tobacco mosaic virus, etc)

5. Biotechnological procedures

Based on the physico-chemical items of the biological reaction of individuals and communication of homogeneous organisms. They are to be applied in combination with traps of different construction for monitoring the population as well as for direct pest control.

5.1. Light traps

With this method the positive photo-taxic reaction of the insects to the source of light in the trap are used. Thus these traps, which are latticed with electrostatically loaded material, are used for the control of the wasps (*Vespa frame*), houseflies (*Musca domestica*),etc. which attract the insects by UV lamps and kills them by contact to lattice.

5.2. Color traps

These traps find very broad use for the monitoring of the pests by the optical attractions of the harming insects on the chromatic spectrum of the light. Thus you take the sticky yellow traps for early recognition and mass catch of the white fly *Trialeurodes vaporariorum*, aphid *Myzus persicae*, cherry fruit fly *Rhagoletis cerasi*; the white traps against wasp *Hoplocampa testudinea*, and the red traps against *Rhagoletis pomonella*.

5.3. Pheromones

Pheromones are highly specific and one of the most important communication signals of homogeneous insects. The chemical identifier of the structure and synthesis of sexual as well as aggregation pheromones opened a new application possibility of the biotechnological procedure with the control of the pests.

5.3.1. Monitoring traps

Monitoring traps are to be used for the prognosis of development of the population as well as for limitation of the treatment. Only a very small dose (0.5-1 mg) of the synthetic substance is needed and due to the number of caught pests the activity of the individuals can be observed.

5.3.2. Masstrapping

Are to be used for the reduction of the pests population. For these purposes 5-50 traps per hectar are divided evenly, which either attract either males (sex-lure) or males and female (aggregation substances). This procedure comes into operation with the fight against bark beetle *Ips typographus*, beetle *Rhynchophorus ferrugineus* etc.

5.3.3. Confusion method

This method finds the broadest application, where the high-dosage (50-250 g/ha) pheromon-sources (capsules, dispenser, donor,carrier) are distributed evenly within the biotope in order to irritate the males. Due to the overload of receptors, the males cannot assume the natural signals sent by the females and therefore, without copulation they die within some days. Thus the commercial products, like " Gossyplur " against red cotton cap worm Pectinophora gossypiella; " Codlemone " against apple rewinding stand Cydia pomonella; " Rak-1... 6 " against grape/cluster rewinding stands Lobesia botrana, apple skin rewinding stand Adoxophyes orana; Synanthedon myopaeformis are successfully assigned.

The insects are classified as harming organisms only if they exceed the certain damage threshold by the rising population density and strongly influence biological production of the cultivated plants.

In order to minimise the damage to the cultivated plants, precautions are taken in order to quickly reduce seizes of pest population below damage threshold.

Conventionally controlling programs using chemical-synthetic means count among them as well as integrated procedures, which combine economic, ecological and toxicological methods. For quite some time the pure ecological plant production gains importance as it does abandon the use of any inorganic means.

As the environment suffers from conventional programs in many cases, the integrated method consist of a link of individual measures, like:

1. selection soil and climate,

2. culture procedure (sort selection, crop rotation),

3. balanced plant nutrition,

4. plant protection (field hygiene, plant protection and pest control by means of physical (mechanical interventions), chemical (direct use of chemicals), biotechnological (traps with signal materials), biological (use of beneficials), genetic (resistance induced) treatment,

which leads to the increase of the plant production on one hand and a indulgence of the environment on the other hand.

PHEROMONES - ALONG WAY FROM IDENTIFICATION TO PRACTICAL APPLICATION

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A general overview is given about the most common techniques for identification of pheromones ranging from the methods of the pioneers up to state of the art methods of today. Various EAG setups are described and obtained data are discussed. The synthetically produced pheromones have to be tested and their efficacy evaluated. Very useful data can be obtained in wind tunnel experiments. Additionally the right choice of dispenser and trap type is essential for a successful application.

General aspects - What are pheromones?

In the view of an entomologist pheromones can be separated in different categories: Sex pheromones released by an individual insect to locate a partner for mating. In most cases the females are releasing the attractive compounds. Male insects (i.e. moths) are able to detect the very special and species specific “perfume” over a distance of up to several kilometres. Aggregation pheromones can be produced and detected by both sexes of a species. They can be used to give information about the location of food sources, places for mating, etc.. Insects like wasps are know to release alarm pheromones indicating danger. Ants as an example are marking the path to their anthill using tracing pheromones.

In the chemists view pheromones are nothing but chemicals, produced by insects (or other animals) serving a very special purpose. Thus they can be classified following the general nomenclature. Most of the identified pheromones are long chain hydrocarbons with a number of 10 to 23 carbon atoms. An even numbers is the rule. They are either saturated compounds or are containing up to three double bonds. Triple bonds are the exception. Generally the hydrocarbon chain is unbranched, but branched pheromones can be found as well. Alcohols, aldehydes and acetates are very common amongst pheromones but other functional groups (ketones, acetals and epoxides, etc) are possible also.

The aim, the development of a pheromone product for practical application, can be achieved following different pathways. The classical strategy includes the following steps: collection - (isolation) - identification - synthesis - verification - application. Another possible route includes synthesis - screening/verification - application. Each of these steps is described below.

Isolation & Identification

The very first step in the process of identification is the collection of the pheromone. A simple but laborious method is the extraction of a large number of insects. The extraction is done with organic solvents and leads to a very complicated mixture of pheromone and many other components. Standard methods of analysis like NMR, IR or MS require pure samples, consequently purification is necessary in advance. Physical procedures like chromatography are common but purification using chemical steps are possible as well.

Far less contaminated with byproducts is the raw material obtained by collecting the pheromone that is emitted by the insects into the air. The sampling of airborne pheromone requires specially designed equipment. One possibility is the collection of the pheromone of a large number of insects “living” inside a closed system. The air that flows through the box is lead through a cold trap where the volatile components are condensed (Fig. 1). Also the sampling of pheromone released by one individual insect is possible. Absorber materials are used to accumulate the molecules (Fig 2).

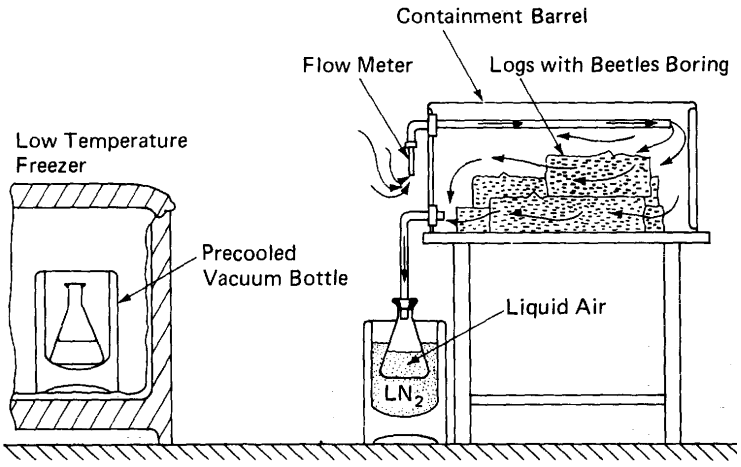

Fig.1 Sampling of airborne pheromone from bark beetles

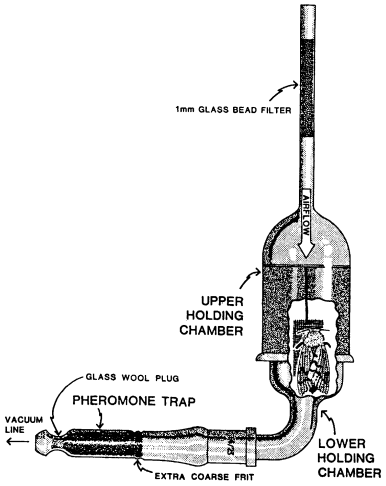

Fig.2 Individual sampling

For further purification of these small amounts preparative gas chromatography is widely used. Analysis is done by MS, IR and NMR afterwards. The GC-MS coupling method needs less material but the obtained spectra is likely to be insufficient for characterisation of a pheromone.

Electroantennogram, EAG technique

The electroantennogram technique makes use of the sensitivity of the insects antennae which are used as detecting device. With their organs some insects even can detect single molecules of a pheromone and the sensitivity is very compound specific. Individuals of an insect only detect the pheromones of their own species and not those of other insects whose pheromones might be similar.

The principle of the EAG method is based on electrical impulses from the neural system of the insect antenna. The antenna is attached to electrodes (Fig 3). Upon contact of the antenna with pheromone electrical impulses can be observed after amplification. The intensity of the signal is related to the amount of pheromone. EAG detectors are more sensitive than conventional detectors (FID, UV, etc).

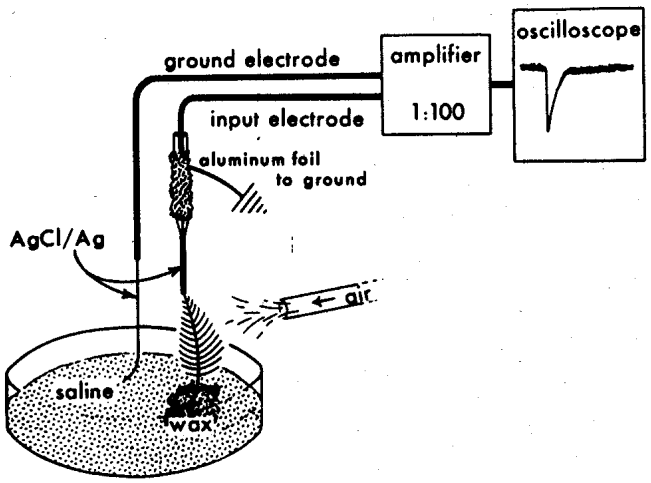

Fig. 3 Simple EAG setup

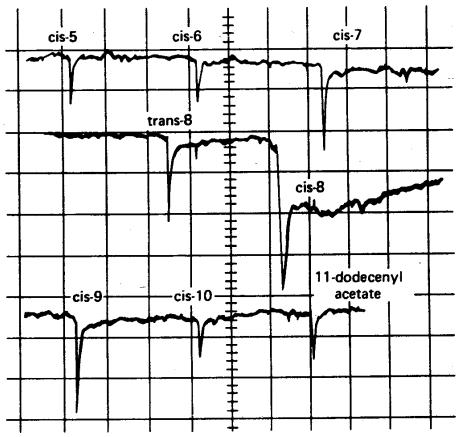

Fig. 4 EAG of *Grapholita prunivora*, (Z8-12Ac)

The EAG spectra obtained with an antenna of *Grapholita prunivora* (Fig. 4) clearly shows the specifity of the method. The Z8 compound causes the signal with the highest intensity. Similar components are detected but the corresponding peaks are smaller. The insects antenna can even distinguish the E8- and Z8-dodecenylacetate, a task not trivial to solve with classical analytical methods.

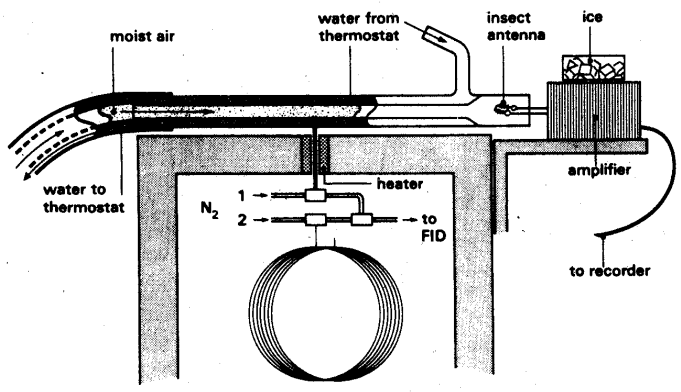

Fig. 5 GC-EAG combination

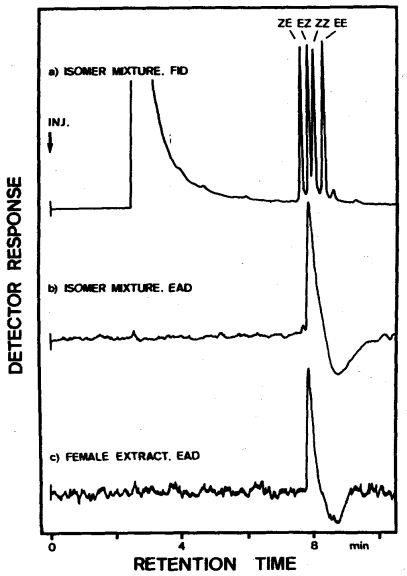

Fig. 6 GC-EAG of *Lobesia botrana*, (E7Z9-12Ac)

In pheromone research the combined GC-EAG technique has proven to be a very helpful and convenient method. At the end of the capillary column of a standard gas chromatograph the gas flow is split in two. One part is directed to the FID detector of the GC the other part is lead over the antenna of the EAG detector. The obtained spectrum provides information about the retention time, FID and EAG activity of a compound. Not only pure compounds can be investigated but mixtures also. Active pheromone components (detected by EAG) in mixtures can be identified by comparing retention the times with those of known pheromones or chemicals in general.

Synthesis & Verification

After a pheromone is identified and all data about its composition and structure is available synthesis is the next step. Several pheromones can be produced easily by using standard reactions. Synthesis of more complicated and enantiomeric pure pheromones might turn out to be a challenge for the chemist.

The synthetically produced pheromones have to prove whether they are indeed identical to natural pheromone, meaning their bioactivity and attractivity has to be tested. In the laboratory the response of the insects' antenna can be shown in the elektroantennogram. But a response does not necessarily mean, that the tested compound will attract insects in the fields as well.

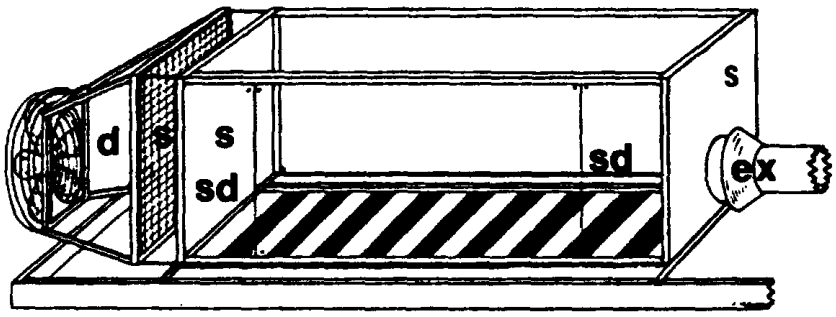

Fig. 7 Scheme of a windtunnel

Fig. 8 Moth in windtunnel experiment

Field conditions can be simulated in windtunnels, closed systems with a constant flow of air where wind velocity, humidity, temperature and other parameters can be modified. Insects flying upwind can easily observed, their behavior approaching a pheromone dispenser mounted inside the chamber can be studied for example. Windtunnels are useful for pheromone screening, testing of synthetical dispensers, verification of analytical results, etc..

Application

The most important types of application are used in practice are monitoring, mass trapping, attract and kill and mating disruption. The different applications and also the

different insects and different climatic conditions require different trap designs (delta trap, funnel trap, wing trap, pitfall trap, color traps and others) and different types of lures (like rubber dispenser, sacchet dispenser, cardboard dispenser and vials).

Parts of information and figures taken from HANS E. HUMMEL, THOMAS A. MILLER, “Techniques in Pheromone Research”.

WHAT WE NEED TO PUT PHEROMONES TO WORK?

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1. Introduction

Vegetable growers historically have been cautious about adopting the use of new technologies. Their crops are frequently of high value and short duration and require exceptionally high cosmetic standards. As a result, unless there is a clear economic benefit, most producers are slow to incorporate new procedures; the adoption of pheromone based technology for insect monitoring has been no exception. However, the use of pheromones in vegetables has been further inhibited by a series of recurring difficulties. Initially the user is faced with decisions regarding trap selection and placement, confusion over best pheromone dose for longevity, and a host of conflicting claims by industry representatives. Then there is the revelation that trap catches, more often than not, do not reflect larval populations in the field. Weather, stage of crop growth, and horticultural practices produce variable impacts on the reliability of pheromone trap data. Nontarget pests sometimes confound the trap collection information and even occasionally represent a health threat. Given these problems, the real surprise is that pheromones have now begun to achieve widespread use in commercial vegetable production for such diverse practices as documenting flight phenology, setting economic injury levels, and monitoring for pesticide resistance.

Insect pheromones offer exciting examples of pure science applied to human benefit. Even before the first pheromones had been identified, the potential for practical exploitation was recognized a potential that has now been realized in many successful schemes using pheromones for direct control of insect pests. Although most uses have been in agriculture and forestry, manipulation of vector insects with behavior modifying chemicals looks increasingly possible in medical entomology for example, via the sex pheromones of sandflies (e.g., Hamilton et al. 1994) and oviposition attractants of mosquitoes (e.g., Beehler et al. 1994; Mordue et al. 1992). There still remains the question why the application of pheromones has not been greater.

Each pheromone meeting since the 1960s has emphasized the potential for pest control. However, we should remember that it is still less than 40 years since the first chemical identification of an insect pheromone and that we have made massive strides since then. Far from being a time for pessimism, I will argue that the basis for optimism is strong and there should be a big push for support. Compared with

conventional pesticides, pheromone research has been only modestly funded. The rationale for increasing funding on pheromone research includes the negligible environmental impact of pheromones and increasing evidence of their effectiveness in pest control. Nonetheless, pheromones represent only 2% of the dollar sales of insecticides worldwide (1985 data in Jutsum 1988), despite great efforts on the part of researchers, companies, and extension workers. How far can slow deployment be blamed on unanswered questions in the scientific understanding of pheromone communication as opposed to practical difficulties in implementation? I will return to each of these topics.

Much of the above could have been written any time since 1960, so what makes now a good time? In part this is due to recent significant advances in pheromone research, particularly in our understanding of orientation mechanisms (Cardé and Knols 2000) and in our ability to measure pheromone concentrations in the field. Developments in neurobiological understanding are also promising. However, equally important are changing attitudes to pest control, by both growers and the public.

2. Current Success of Pheromones

We have come a long way since Rothschild (1981) said that "pheromone researchers... will have to accept that it may be some years before disruption is accepted as a viable control method for any more than 1 or 2 species." Cardé and Minks (1995) review some of the major successes of direct control of moth pests by pheromones in field crops and orchards: commercially viable formulations as effective as, or better than, the conventional insecticides they replace. Borden (1997) reviews a similar picture of success in the use of pheromones for control or monitoring of forest pests, both beetles and Lepidoptera.

2.1. Using Pheromones: Monitoring and Control

The focus of this section is on using pheromones for direct control of insect pests, but a major use of pheromones continues to be for monitoring pest populations both of crop and orchard pests (Wall 1988), stored products (Burkholder 1990), and forestry pests (Borden 1997). Pheromone based monitoring provides one of the most effective survey methods for detecting pest species. Trumble (1997) describes some of the persistent problems with monitoring as a quantitative decision tool in particular the low correlations between adult male trap catches and caterpillar levels in the crop. However, more sophisticated schemes incorporating decision thresholds have been tried with some success, and significant reductions in pesticide use can result (Wall 1988).

Pheromones have been used for direct control of insect pest populations in three main ways: mass trapping, "lure and kill" (including "push pull" mechanisms), and mating disruption. Much of what follows emphasizes control of moth pests by mating disruption through use of synthetic sex pheromones released in the field to permeate

the crop or provide numerous point sources of synthetic pheromone. In pest moths (among the most important of agricultural pests), because it is the larvae which do the damage, the aim of pheromone intervention is to prevent fertilization of eggs by preventing mates meeting. However, direct control in other systems, especially with bark beetles (Borden 1997), does not involve interference with sex pheromones but, instead, involves manipulation of aggregation pheromones and the process of tree colonization, including exploitation of sometimes complex species interactions mediated by semiochemicals. There are many other potential uses of semiochemicals for control (Tumlinson 1988), including marking pheromones such as oviposition marking pheromones in both flies (Prokopy 1981) and moths (e.g., Schoonhoven 1990), oviposition attractants, larval interactions mediated by pheromones, kairomones which modify the behavior of natural enemies (Pickett 1997), and direct control by non pheromone attractants such as male lures for tephritid fruit flies for lure and kill (Cunningham et al. 1990). Reviews of pheromone applications to particular crops can be found in Ridgway et al. (1990). Jutsum and Gordon (1988) and Howse et al. (1996) review applied pheromone use in agriculture more generally.

2.2. How Have Research Agendas Changed?

The use of pheromones as control agents has been reviewed regularly (e.g., Shorey and McKelvey 1977; Mitchell 1981; Jutsum and Gordon 1988; Ridgway et al. 1990). It is sobering to see how little research agendas and concerns have changed since then. Many of the points raised by this researchers are still concerns: the mechanism of mating disruption (involving sensory biology and behavior) and the need to understand the population biology (including migration and other characteristics) of the pest species. Other issues have included the difficulty of evaluating the effectiveness of pheromone trials and practical questions of registration. The major question now must be how to move research and applications forward.

Where initially the challenge was chemical the enormous quantities of material originally needed for analysis are legendary with the integration of gas chromatography coupled mass spectrometry (GC-MS) with gas chromatography - electroantennogram / single sensillum recording (GC-EAG/SCR) and the increasing sophistication of chemical analysis, chemical identification is rarely the rate limiting step. Instead, the persistent problem is the complexity of insect behavior, and in particular our ignorance of behavior in the field. Whereas pheromones can be used for monitoring purposes within a few years of discovery, mating disruption takes much greater understanding.

3. Progress in Understanding Mating Disruption: Successes and Problems

Since the effectiveness of direct control with pheromones has been demonstrated in many systems, one could argue that we do not need to understand the mechanisms. It may seem a luxury, but as Sanders (1997) points out, to improve methods and understand how to change them when they fail, we need to know how they work. Such an understanding would also help in predicting likely resistance mechanisms (although insects are likely to surprise us). Much of the discussion that follows is devoted to mating disruption in moths, but many of the methodological problems are relevant to all pheromone mediated control systems.

3.1. Poor Understanding of the Mechanisms of Mating Disruption

In most species, males find the female by flying upwind in response to her pheromone. To prevent successful mating, a number of stages in the mate location and courtship sequence could be interrupted, but long range orientation may be the link most vulnerable to interference from synthetic pheromone because short range orientation and courtship may involve other sensory cues (Sanders 1997). Mating disruption describes the result not the mechanism(s) by which this is achieved.

Sanders (1997) and Cardé (1990) review the supposed mechanisms, much as Bartell (1982) proposed: (1) sensory adaptation (at the peripheral receptor level) or habituation in the CNS (lumped as "sensory fatigue" in Sanders), (2) false trail following (competition between natural and synthetic sources), (3) camouflage of natural plumes by ubiquitous high levels of synthetic pheromone, (4) imbalance in sensory input by massive release of a partial pheromone blend, and (5) the effects of antagonists to attraction and pheromone mimics.

As Sanders explains, field results combined with laboratory wind tunnel tests and electrophysiological recordings can sometimes suggest the main mechanisms which might be involved in particular situations but given the variety of proposed mechanisms, the real breakthrough will be to design critical field and laboratory experiments to distinguish the hypotheses. However, even in one species, many mechanisms may be acting, perhaps synergistically (Cardé 1990).

3.1.1. Orientation Mechanisms and Mating Disruption

The unifying paradigm is the growing understanding of how males track upwind toward an odor source (Baker 1990; Mafra-Neto and Cardé 1994; Vickers and Baker 1994), building on the work of the last 25 years. The link between male behavior and the filamentous structure of odor plumes (Murlis et al. 1992) has been clarified. The key features seem to be the male's brief surge upwind in response to each antennal contact with filaments of pheromone laden air and casting on its loss.

The male's response to pheromone "filaments" helps explain why the natural blend is often the most effective for mating disruption (Minks and Cardé 1988). Vickers and Baker's (1994) wind tunnel experiments show that the individual components of multicomponent blends (the norm) need to be in the same puff for optimum upwind flight, in line with other behavioral work showing the importance of the full blend at all distances from the female (Linn et al. 1986). Nonetheless, some mating disruption systems have used off blends successfully (Sanders 1997).

Sanders (1981) observed that in field trials, "inhibitors" were only effective if released very close to the female (see also Cardé 1990); he suggested that this was due to the structure of plumes, as above: Only if superimposed on the pheromone, in the same filaments, would inhibitory compounds work. Recent laboratory work supports this (Liu and Haynes 1992; Rumbo et al. 1993). Although inhibitors may offer control in some cases (e.g., Bengstsson et al. 1994), the prospect is not generally promising.

3.1.2. Primer effects in Mating Disruption

Most behavioral work on mechanisms has understandably focused on the immediate (releaser) effects of synthetic pheromones during the males' search for the female. Much of the effect could be due to the longer-term (primer) effects of permeation of the field with pheromone during the rest of the day (or even the period of activity immediately preceding flight). Despite pioneering work by Bartell (review 1982) and later work (e.g., Linn and Gaston 1981; Liu and Haynes 1992), the effects of pre-exposure of different types and duration are not clear. There is a pressing need to expand the timescale and range of experiments to investigate the more subtle primer effects.

Primer effects also reinforce the need to know where resting animals are. For example, during the day, pink bollworm male adults go down to the base of the plants into the soil and may avoid exposure to the pheromone in the air (Flint and Merkle 1983).

3.1.3. Wider Taxonomic Variations: Generalizing to Other Insect Orders

While our understanding of moth orientation increases, an underlying question remains about how far these inflight mechanisms apply to other insect groups such as beetles, sawflies, and now aphids (Pickett et al. 1997) which also use long distance pheromone communication (Cardé 1990). All moths, through common ancestry, might well share the same mechanisms, but other taxa could have evolved orientation behavior independently. This is important because moth orientation models inform much of the theoretical discussion of disruption. The same question may apply to other pure and applied aspects of mating disruption.

3.1.4. Tracking Individual Moths in the Field

Currently a major handicap is that it is not usually possible to track individual moths in the field, except for short distances close to the pheromone source in experiments (e.g., Willis et al. 1994). An unanswered question is the behavior of males before first

contact with the pheromone. Could radiotracking yet comewithin reach for the larger moths, as miniaturization of electronics continues? This technique would also help answer what the animals do during the day.

3.2. Pheromone Concentrations in the Field and the Structure of Plumes

One of the major unknowns in pheromone research is now partly solved: New portable electroantennogram (EAG) and single sensillum recording (SCR) equipment mean that we can now measure instantaneous concentrations of pheromones in the air. As recently as 1990, Am wrote that the invention of a "sniffer" to monitor concentrations continuously in the field would be "a golden key to pheromone applications" (Arn 1990). Earlier techniques could only average over a longer period by adsorption on a pheromone collector because air concentrations are too low for instantaneous measurement by GC-MS. The new systems exploit the sensitivity and specificity of the moth's antenna to its own pheromone and combine this with a mechanism for'calibration of the EAG or SCR responses. Whether EAG or SCR is the best technique may depend on the question.

The new techniques will help in two ways first, by allowing the fine structure of pheromone plumes to be measured directly rather than by proxy of ionized air technique. Already these studies confirm earlier results on plume fine structure (Murlis et al. 1992). Second, rather than speculating, we can now investigate the concentrations (and the spatial and temporal patterns) of airborne pheromone which different mating disruption strategies produce. Similarly, the effects of adsorption of pheromones onto leaves and the effects of the canopy on air movements can be investigated (Suckling and Karg 1997). We can now start to measure the "active spaces" of plumes downwind of lures in traps to study trap interference in a new way.

3.2.1. Wind Tunnel Concentrations

The new technologies for pheromone air concentration measurement now mean that laboratory studies can be made more realistic. Until now, pheromone concentrations have usually been given as dose on a source rather than the achieved air concentration.

3.3. Complete Blends and Why They Matter

While pheromones for large numbers of insect species have been identified, it is likely that many earlier identifications are incomplete (Tumlinson 1988). As Borden (1997) points out, mating disruption projects abandoned in the past might have been using incomplete blends. In some of these trials, real females outcompeted synthetic sources implying that suboptimal pheromones were being used. It would be worth going back to many of these with a fresh identification of the blend. The role of host plant volatiles as synergists with insect pheromones is also being increasingly recognized for some moths and bark beetles (Borden 1997).

Producing effective mating disruption schemes is further complicated by the existence of geographical races using different blends (isomers, enantiomers, ratios) for example bark beetles (Lanier and Jones 1985) and moths such as the European corn borer *(Ostrina nubilalis)* and turnip moth *(Agrotis segetum)* (Löfstedt 1993).

3.4. Delivery of Pheromones in the Field

McLaughlin and Mitchell (1981), for example, argued that a lack of reliable pheromone delivery systems was a major problem. Since then, many new systems have been developed (Jutsum and Gordon 1989; Leonhardt et al. 1990), but providing constant release (zero order) systems remains a problem. Quality control can also be patchy.

Sanders (1997) discusses the likely effects of different formulations on mating disruption. In some ways, only now does it make sense to make things more sophisticated because now we can measure the concentrations produced (above). A new challenge for dispenser technology comes from our recognition of the need for release blends close to the natural one (Tumlinson 1988).

4. Putting Pheromones to Work: Progress and Problems with Applications

4.1. Barriers to Take Up

While most of this chapter has been about science, it will not surprise the reader that, in common with much of IPM (Dent and Pawar 1991), the most serious challenges to implementation of pheromones in pest control are probably political and economic, cogently reviewed by Silverstein (1981). Moving from the science, or even research field trials, to take-up by farmers is the most difficult step (Dent and Pawar 1991). Each review over the last 25 years has identified the problem which, as the characteristics of pheromones remain the same, have changed little. Here I will focus on some of the principal ones and suggest some ways forward.

4.2. Who Pays

The problems of commercial development have been rehearsed many times. It was recognized early that it is unrealistic to expect the large agrochemical companies to develop commercial pheromone technologies for other than a very few major crops, notwithstanding the significant involvement of some large companies (Jutsum and Gordon 1989). The barriers to commercial interest mostly concern the problem of recouping high development costs. They include small markets (and thus relatively high unit cost of active ingredients), formulation difficulties, marketing and technology transfer difficulties, problems with patentability (Silverstein 1981) to which Arn (1990) added the difficulties presented by increasing complexity of pheromones, in both chemistry and biology, and, for pesticide companies, competition with their own conventional products.

There are strong parallels with biological control which shares many of the same barriers to commercial development (Jutsum 1988). The opportunities for recovering development costs commercially may be limited, but government or similar public sector investment can be more than justified by the public benefits: to individual farmers, to consumers, to the environment, and (for Third World countries) in savings of foreign exchange. It is always easy to argue for more funds, but here is a case where public investment would pay dividends: Not all "public goods" come from choices dictated by return on capital narrowly defined by company interest. This comment may seem overpolitical but reflects commercial realities.

Most companies involved in commercialization of pheromones, which tend to be small, do not have the resources for the basic research needed in developing the technology. Much of the research and extension work will need to be government-supported. However, as Jones (1987) points out, it is ironic that at the same time as politicians are legislating for reductions in conventional pesticide use (below), they seem most keen to reduce the worldwide capacity for scientific research into alternative pest control methods at government and academic institutions. The trends in many western countries toward commercialization and privatization of government research (e.g., HortResearch in New Zealand, CSIRO in Australia, and GHRI and NRI in the United Kingdom) do not bode well. Even today, current investment is small. Van Lenteren and Woets (1988) estimate that the amounts spent on biological control, largely in the public sector, represent about only 1 % of investment by chemical companies in pesticide research. Levels of investment in pheromone research are likely to be on the same order.

Campaigning for increased investment will need readily accessible (and up to date) information, including successes (e.g., Cardé and Minks 1995), current usage, costs, and benefits. Without political lobbying, it is unlikely that we will have progress with pheromone adoption. A major handicap is the lack of powerful equivalents to chemical industry organizations, although with the formation of the American Semiochemical Association and European Semiochemical Association its a good beginning.

Changing consumer attitudes toward pesticide use are already having effects on the climate for alternatives to conventional pest control, and pheromones are likely to benefit. Other incentives for the development of mating disruption and other IPM techniques come from growing insecticide resistance and the rising costs of developing new pesticides.

4.3. Extension, Persuasion, and Take-up

Gaining acceptance for pheromone based pest control, like adoption of other new IPM methods, requires considerable effort. The description of the conservatism of vegetable growers and their reasonable suspicion of new pest control technologies (not all have matched their promise) is typical. Adoption will be more likely if it is clear

to farmers that there *must* be change, in response (for example) to pesticide resistance problems (e.g., Suckling and Karg 1997). Getting acceptance of ultimately better but less certain pest control methods is difficult. Schemes requiring much greater sophistication on the part of farmers will be less successful than those incorporating a more sympathetic appreciation of farmers' perceptions and constraints. Extension workers need to match the pesticide company sales effort at the farm level. The chances of adoption will be improved if mating disruption techniques can be seen to be cost-effective in schemes involving farmers.

Some of the most effective uses of pheromones for mating disruption have involved region wide schemes (e.g., Campion 1984; Jones 1987). Once implemented, involvement of the government can ensure adoption across the whole region, but getting the initial decision can be more difficult than dealing with individual farmers.

Investment in implementation, the key to adoption of pheromone technology, is often skimped, or not properly addressed at all. Unfortunately, this crucial activity is one of the most dependent on public funds.

Pheromones could be very appropriate technology for developing countries, but Silverstein (1990) reports excellent critiques of many current approaches and emphasizes the need for ingenuity and simplicity in the integration of pheromones into pest control systems.

4.4. Selecting Good Target Species for Mating Disruption

Rothschild (1982) feared that there would be few "universal truths" that can be applied to mating disruption, which would mean that each pest species (and crop system?) might have to be studied individually. To make the task manageable, we need to search for patterns, good predictors of success (or likely failure) in pest species and agricultural systems, resting on basic studies on field behavior, pest biology, and mating disruption experience. Testable suggestions for such characteristics have been made (e.g., Rothschild 1982; Sanders 1989). This challenge should be taken up.

4.4.1. Pest Complexes

A major strength of pheromones is their effectiveness as part of IPM schemes, because of their compatibility with biological control agents and other beneficials. Trumble (1997) describes use of pheromones in IPM of tomato pests in Mexico. Pheromones fit well in the virtuous spiral for example, in greenhouse IPM (Van Lenteren and Woets 1988), where the use of one biological control agent encourages (or requires) moves from conventional pesticides for other pests.

4.6. Chemical Advances

Costs of active ingredients have been a limiting factor in some mating disruption schemes. Developments in synthetic chemistry in particular, chiral synthesis (driven

by the pharmaceutical industry after thalidomide) may bring down costs. The use of biotechnology for pheromone synthesis could produce biologically correct pheromones cheaply (Lindgren 1990; Pickett et al 1997).

5. Conclusions

Butenandt could have had no idea in 1950, contemplating 500,000 moths to identify the silkworm pheromone, bombykol, that today such a task could be attempted with a single female. Similarly, the idea of measuring pheromone concentrations in the wind would have seemed a fantasy. Problems with mating disruption remain, but in many cases we now have the tools to answer them. Finding the answers needed to make pheromones an even more effective weapon in the direct control of insect pests will be of mutual benefit to both pure and applied biologists.

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NEEM IN BRAZIL – PLANTATIONS, EXTRACTS, RESEARCHES AND MAIN PESTS WITH POTENCIAL OF CONTROL

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Insect pests are responsible for severe economic losses in Brazil and in the world. In the first half of the 20th Century, Brazil was a great producer and exporter of botanical insecticides, like rotenoids (*Lonchocarpus* sp. and *Derris* sp.), pyrethrum (*Chrysanthemum cinerariaefolium*) and nicotine (*Nicotiana tabaci*). However, following the international trend after the 50s, the control of insects was mostly performed with chemical synthetic compounds. The negative consequences of the excessive use of these compounds to health and to the environment are well known. In Brazil, the high costs and economic dependency on foreign products can be also considered. Some alternative methods, like biological and cultural control or the use of pheromones started to be studied and used in few situations in the last decades. More recently, the interest of consumers and producers towards organic products, free of chemicals, is increasing sensibly, so leading to the search of new alternatives.

The neem products are quite promising because of their low price, ease to prepare, action over more than 400 species of insects, multiple action, reduced effect on beneficial insects and because they are approved worldwide to be used in organic farming. Neem was first introduced in Brazil to be studied as insecticide in 1986, by IAPAR. Although having tropical origin, the species has developed well in subtropical areas of South of Brazil. The production of fruits, however, was very low. During the last decade, the interest on neem production has increased, due to its wide possibilities of economic exploitation, like agricultural production of seeds, fruits and leaves (row material for extracts), wood production, industrial production of different sorts of extracts, oils, cakes, shampoos, repelents, creams, etc. to be used in agriculture, and also in human and animal health.

There are above 150 thousand trees planted in different counties, mainly in Goiás, Tocantins, Pará, Distrito Federal, Bahia, Espírito Santo, São Paulo, Paraná. All these plantations are young, mostly no older than three years. Many areas are established for reforestation purposes. The interest in planting neem is increasing all over the country. There is a great variability on the growth and development of the plants in the plantations, due to the absence of breeding programs or even seed selection. Also, the plant development and fruit production vary with the climate, soil type and altitude. Fruits are produced in December/January in Centre and Northeast from Brazil, March in Southeast areas and later, in May/June, in South of Brazil. Several neem producers prepare and sell neem extracts and neem oil in small scale,

although there is not any registered product in Brazil yet. Most of the emulsionable neem oil is prepared by importing crude neem oil and mixing with emulsifiers. The market for these products is still being established and the demand increases every day. However these products need to have their technology of production and stability improved and need to be registered. Besides, more studies on their use as control agent have to be carried on.

The scientific research on neem in Brazil has spread out more recently attending to the demands of farmers and consumers. In IAPAR, the action of neem extracts and neem oil has been studied on pests of economic importance in agriculture. Some findings are: larval mortality of *Spodoptera frugiperda* larval mortality, prolonged development, reduction of food consumption, reduced fecundity of *S. littoralis*, mortality of the mites *Polyphagotarsonemus latus*, *Tetranychus urticae*, *Brevipalpus phoenicis* and *Phyllocoptruta oleivora*, oviposition repellence and ovicide effect on *Leucoptera coffeella*. Treated leaves promoted reduced consumption and mortality on *Diabrotica speciosa*. Sprays on the predator *Cycloneda sanguinea* have shown the neem oil ineffective on adults, but the recommended dose (5ml/l) killed 40% of the larvae, although caused no alterations in the development or in pray consumption. Other research institutes and universities have included neem studies in their research program, like Universidade Federal de Viçosa/MG, Universidade de Goiás/GO, Embrapa arroz e Feijão/GO, Universidade Estadual de Londrina/PR, Embrapa Tabuleiros Costeiros, Embrapa Milho e Sorgo/MG, Universidade Federal Rural de Pernanmbuco/PE, etc. It was shown that neem oil caused mortality of *Plutella xylostella* in Brassicae, and reduced egg viability and caused mortality of the mite *Mononychellus tanajoa*; leaf extracts caused larval mortality of *S. frugiperda* in corn. Besides, in store beans, neem oil caused mortality and prevented the development of populations of *Calosobruchus maculatus.* The use of neem extracts to control ticks on cattle are also being studied.

IAPAR is trying to obtain plants more adapted to subtropical conditions by means of importing neem seeds originated from the same conditions and by testing neem grafted on *Melia azedarach*, another Meliaceae, highly productive and fast grower in subtropical conditions. Variations in azadirachtin content during plant phenology and in extracts stored under different conditions are also under evaluation.

Brazilian situation and experiences indicate a great potential of production and use of neem based technologies, mainly due to the wide possibilities of economic exploitation of the tree, to the wide areas with climate favorable to plant neem trees and to the increasing demand of neem products.

NEEMAZAL-T/S: UM PRODUTO NATURAL PARA CONTROLE EFICIENTE DE INSETOS PRAGA

NEEMAZAL-T/S A BOTANICAL PRODUCT FOR EFFICIENT CONTROL OF INSECT PESTS

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Properties of an extract "NeemAzal" obtained from seed kernels of the tropical Neem tree Azadirachta indica *A. Juss and its formulation are described. Toxicological and ecotoxicological aspects of its use are described and the mode of action is discussed.*

Neem,* Azadirachta indica, *Azadirachtin, toxicology, ecotoxicology, mode of action

The Neem tree

Different parts of the tropical Neem tree *Azadirachta indica* A. Juss are used in India since times immemorial for curing many diseases (Ketkar and Ketkar, 1993). In a holistic perception the protection of plants and animals against diseases and illness is a medical issue as well. The leaves and especially the seed kernels of the Neem tree and their extracts have been used for the control of various insect pests in India. On behalf of different reasons there is a demand for standardised natural products for plant protection today. Our research has combined the experience of the thousands years old Indian experience and modern demands for plant protection products. The result of our development is the formulation NeemAzal^{TM}-T/S.

What is NeemAzal-T/S?

NeemAzal-T/S is a formulation of the highly concentrated active ingredients of the Neem-tree, namely the Azadirachtins (Devakumar, 1993; Kraus, 1995). This concentrate – named "NeemAzal" – contains in an average 34% AzadirachtinA, about 20% other Azadirachtins (Kleeberg, 2001) and 46% of inert ingredients like lipids, oligosaccharides, hydrate water. NeemAzal is formulated with the help of surfactants (produced from renewable resources) and edible plant oil to obtain an emulsion concentrate EC containing 1% AzadirachtinA.

Physico-chemistry and degradation

The formulation has a shelf life of more than 2 years if stored below 20°C in a dark place. It forms a stable emulsion with water and spreads easily for example on leaf surfaces. An octanol-water distribution coefficient below 10 (Ruch et al., 1997)

indicates a low potential for accumulation in fatty tissue and hence in the food chain. Azadirachtins are not much adsorbed by soil and thus leach rapidly (Ruch et al. 1997). However, the (especially microbiological) degradation is very fast, so that a risk of contamination of ground water can be excluded (Ruch et al., 1997). In water NeemAzal is transformed very rapidly by light (degradation half life about 10 hours) (Ruch et al., 1997; Troß et al., 2000; Pussemier, 2000; Michalski, 2001). After spray application to leaves and fruits AzadirachtinA is degraded rapidly with a half life of the order of very few days (Troß et al., 2000, Ruch and Kleeberg, 2001).

Toxicology

NeemAzal and NeemAzal-T/S have been investigated thoroughly with respect to possible toxicological impacts to mammals. Neither acute nor subchronic or chronic studies indicate the presence of important risks for humans or mammals (Stewart, 1998, Niemann and Hilbig, 2000, Niemann, 2001). This is especially established with respect to carcinogenicity, teratogenicity, reproduction etc. In this connection it is important to state that these “non-toxic” properties refer only to the concentrate NeemAzal and its standardised formulations and not to other “Neem-products” since these may have considerably different compositions.

Ecotoxicology

NeemAzal-T/S has been studied carefully with respect to possible side effects on the environment. Table 1 summarises results obtained for aquatic organisms. The high “No Observable Effect Concentrations” NOEC indicate an extremely low risk to aquatic organisms; this is true especially in view of the low concentrations of AzadirachtinA which are necessary for efficient applications (i.e. of the order of 15 to 30 ppm AzadirachtinA in typical spraying solutions (see table 1).

Table 1: Summary of the aqua toxicological results for NeemAzal and NeemAzal-T/S

Organism	Test substance	NOEC mg/l *	NOEC mgAzA/l**	Time of exposure
Algae	NeemAzal-T/S	22	0,22	72 hours
Daphnia				
Daphnia magna	NeemAzal	2,5***	0,74	21 days
Daphnia magna	NeemAzal-T/S	6,25 reproductive output	0,06	21 days
Fish:				
Rainbow trout	NeemAzal-T/S	100	1	96 hours
Freshwater carp	NeemAzal-T/S	100	1	96 hours
Zebra fish	NeemAzal	6,4***	1,9	1.5 life cycle (~ 7 months)
Rainbow trout	NeemAzal-T/S	75	0,75	28 days

*NOEC: No Observable Effect Concentration, **NOEC-value of the test-substance converted to AzadirachtinA concentrations, *** highest concentration tested

Obviously microbiological organisms degrade NeemAzal-T/S rapidly. This may lead to peculiar effects: for example the activation of the soil microfauna leads to an increased weight gain of earth worms after application of NeemAzal-T/S (Ruch et al., 1997).

Beneficials are generally not influenced to a meaningful extent by NeemAzal-T/S applications (Forster, 2001) - with the exception of thin skinned species (like syrphids) (Hermann et al., 1999).

Acute as well as reproduction studies with honey bees show (Leymann et al., 2000) that no adverse effects may be considered after application of NeemAzal-T/S.

Studies on chicken as well as field observations do not show any significant effects with respect to birds.

Residues

The fast degradation of Azadirachtins on/in plants, the low amount of the active ingredient applied per hectare and the favourable toxicological properties indicated that even very short time after the application of NeemAzal-T/S residues can not be considered a problem. Analytical investigations indicate that the concentration of residues depends on the surface area to mass ratio of the treated crops. Thus for example AzadirachtinA residues in/on leaves are significantly higher than on apple or tomato (Ruch and Kleeberg, 2001).

Mode of action

After the treatment with NeemAzal^{TM}-T/S larvae react with feeding and moulting inhibition and mortality; adult (beetle) show feeding inhibition, infertility and to a lesser degree mortality (Kleeberg, 1992; Otto, 1994; Hummel and Kleeberg, 1996; Hummel and Kleeberg, 1997; Schulz et al., 1998).

As a result of this comparatively slow **„insectistatic“ mode of action** of NeemAzal^{TM}-T/S a final assessment of the treatment should be done 7-10 days after application under practical conditions. The number of dead pest insects is not necessarily a good evaluation criterion. For the assessment the following criteria are appropriate: loss of leaf mass, damage to plants, formation of honey dew, crop yield, development of the pest population, positive effects on beneficials (Kleeberg and Hummel, 1999).

Table 2: Time dependence of phenomena observed after treatment with NeemAzal-T/S

Phenomenon	**Timing**	**Description**	**Assessment**
Feeding inhibition	after hours	reduced food consumption	reduction of: weight increase, plant damage, faeces and honey- dew production
Inactivity	after days to 1-2 weeks	over all reduction of fitness, molting inhibition, starvation	mortality
Fertility reduction	after weeks	reduction of progeny (next generation)	reduction of the next population

The success of the application with NeemAzal^{TM}-T/S depends on the progress of the pest infestation and adequate timing of the treatment.

In the case of **a temporary infestation and synchronous development** of pest populations one application per generation or season is generally sufficient (under European climatic conditions, usually one or two generations, for example: appearance of fundatrices of the Rosy Apple Aphid *Dysaphis plantaginea,* first adults of Elder Bush Aphid *Aphis sambuci* (Hom., Aphididae), first young larvae of Colorado Beetle *Leptinotarsa decemlineata*, beginning of flight of Cockchafers (*Melolontha* sp.)).

In case of a **permanent infestation** (several generations like Aphids, Thrips, White Flies, Spider Mites etc.) repetitive applications are required. The interval between treatments is usually 7 to 14 days and depends on climatic conditions and infestation pressure.

NeemAzal^{TM}-T/S is harmless to most beneficials - they are an important factor in the control of the remainder of the pest population. **NeemAzal^{TM}-T/S** can favourably be combined with the use of beneficials in plant protection conceptions.

Phytotoxicity information

NeemAzal^{TM}-T/S was tested with many plants under outdoor and greenhouse conditions and shows generally good plant compatibility during the warm season. The compatibility of **NeemAzal^{TM}-T/S** depends on the variety and species of plant. Some ornamental varieties react with leaf and blossom damages. Some pear varieties react with strong leaf necrosis already from spray drift. It can not be excluded that damage can occur in cases of plants with known good compatibility.

In **ornamentals** the following plants react on **NeemAzal-T/S^{TM}-**treatment with:

good leaf and blossom compatibility - *Antirrhinum majus, Acalypha hispida, Argyranthemum frutescens, Astericus,* Begonia-hybrids, *Bidens ferulifolius, Brachycome,* chrysanthema *(*Merced, Bronze Arola, Kory*), Celosia cristata, Convolvulus sabatius, Coreopsis* (girls eye), *Dendranthema grandiflorum, D. indicum, Diascia, Euryops chrysanthemoides, Fuchsia, F.*-hybrids, *Gazania splendens, Gerbera jamesonii, Glechoma, Helichrysum petiolare, Kalanchoe* (Boston), *Lantana*-Camara-hybrids, *Lobelia*, *L speciosa, Manettia bicolor, Mentha,* Carnations (Aristo), Slipperwort, Pelargonien, *Petunia, Pilea microphylla,* Roses (Komet), *Rudbeckia, Sanvitalia procumbens, Scaevola, Sutera, African marigold* (yellow), *Torenia fournieri, Verbena (Tapien blue)* (Sunvop (P),

good leaf compatibility - *Agerathum houstonianum, Alonsoa, Alyssum, Amaranthus,* Calceolaria hybrids, *Callistephus chinensis, Calocephalus brownii, Centaurea, Cestrum, Clarkia, Cleome, Coleus, Cosmos, Cuphea, Cynara scolymus, Dahlia, Dianthus barbatus, Dimorphoteca, Eucalyptus, Eustoma grandiflorum,* Ficus,

Felicia, Gazania, Gnaphalium, Helianthus, Heliotropium arborescens, Iresine lindenii, I. herbstii, Kochia, Lavatera, Limonium, Lotus, Lysimachia, Melampodium paludosum, Mesembryanthemum crystallinum, Nicotiana, Nigellia, Pennisetum, Penstemon, Plectranthus fruticosus, Polygonium, Portulaca, Ricinus, Salvia farinacea, Saintpaulia (Miho io), *Senecio, Serenoa, Streptocarpus, Tanacetum, Tithonia, Trachelium,* Viola, *Veronica, Zinnia,*

blossom damages - *Begonia* semperflorens hybrids, Chrysanthema *(Deep luv), Euphorbia pulcherrima* (Peter star, Cortez), *Gerbera* (Pretty red, Sigma, Luciana), Impatiens Neu-Guinea hybrids, *Impatiens walleriana,* Pelargonium-Peltatum-hybrid, P.-Zonale-hybrids, *Solanum rantonnetti, Saintpaulia* (Miho io), *African marigold*, Verbena (individual sorts),

leaf damages - Abutilon hybrids, *Cestrum, Datura, Euphorbia pulcherrima,* Impatiens Neu-Guinea hybrids, *Impatiens walleriana,* passion flower*, Solanum rantonnetti,* Roses (Papa Meilland, White Noblesse, Saphir, Ducat, Eveline, Alina, Baronesse, Lola, Black Magic, Noblesse, Roulette, Funcky Jazz, Arabia).

In **orchards** serious plant toxicity has been observed in the case of pear varieties ‘Conference’, ‘Alexander Lukas’, ‘Bristol Cross’, ‘Comice’, 'Guyot’, ‘HW 606’, ‘Illinois 13 bars 83 Maxi’, ‘Lectier’, ‘Trevoux’, ‘Winter dechant’.

In the case of plant species that normally react insensitive, individual varieties can exhibit incompatibilities and it is proposed to perform sensitivity tests with a few plants or some leaves in the respective stadium of growth 3 to 5 days before treatment of larger areas.

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POSSIBILIDADES DE RISCOS DO NEEMAZAL-T/S PARA ORGANISMOS AQUÁTICOS: PEIXES – TESTE DE CICLO TOTAL DE VIDA DO ZEBRAFISH, *DANIO RERIO*.

EFFECTS OF NEEMAZAL ON FISH: FULL LIFE CYCLE TEST WITH THE ZEBRAFISH, *DANIO RERIO*

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Summary:

A study was conducted in order to evaluate the effects of NeemAzal (a standardised variant of the active ingredient Azadirachtin) on different life stages and generations of zebrafish (*Danio rerio*) during a full life cycle, including reproduction. The study provides data being relevant for the assessment of effects on populations. This report deals with the complete life cycle test, including an early life stage test of the F_I-generation (day 1 - day 37), investigations on survival, growth and reproduction of F_I-generation from day 38 to day 135, as well as an early life stage test of the F_{II}-generation (day 135 - day 174). Lethal and sublethal effects were assessed and compared with control values to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC). Early life stage tests with F_I and F_{II}-generation were carried out in accordance with the OECD guideline 210 (fish, early life stage toxicity test (1)). In the study plan of this life cycle test a conception was attempted which may qualify as a general approach to evaluating the effects of substances on the population of fish and their development. The results show that the conception may well serve as guideline. During the course of the study the concentration of active ingredient was monitored at regular intervals analytically. The investigated NeemAzal concentrations were (mg/L): 0 (control), 0.2, 0.63, 2.0 and 6.4, respectively.

According to the obtained results and following the model of a concentration-response-relationship, the following effect concentrations can be stated for the complete full life cycle test (174 days) with zebrafish and NeemAzal as test substance.

Zebrafish, full life cycle test with NeemAzal as test substance,
F_I- and F_{II}-generation (day 0 - day 174):

LOEC (lethal effects):	**> 6.4 mg/l**	(no lethal effects were observed up to the highest test concentration)
NOEC (lethal effects):	**6.4 mg/l**	(nominal concentration)
LOEC (sublethal effects):	**> 6.4 mg/l**	(no sublethal, concentration-dependent effects were observed up to the highest test concentration*)
NOEC (sublethal effects):	**6.4 mg/l**	(nominal concentration)

* = **Remark:** Significant reduction of egg production in 0.63 mg NeemAzal/l, but not in 2.0 and 6.4 mg NeemAzal/l, respectively.

Introduction:

In order to estimate a possible impact of the application of NeemAzal-formulations on fish a GLP-test was designed and conducted where zebrafish (*Danio rerio*) were exposed continuously to four different concentrations of NeemAzal. Since test start, test animals were exposed to four concentrations of NeemAzal with two replicates each, under flow-through conditions. An untreated control with two replicates was run in parallel. The study started by exposing fertilised eggs. The first 37 days of the full life cycle test correspond to the fish early life stage toxicity test (with F_I-generation) in accordance with the OECD guideline 210 (1).

Further on, the fish were exposed until they reached sexual maturity. Mortality, behavioural abnormalities, egg production, fertilisation- and hatching-rates of the produced eggs were recorded. The offspring was used to conduct a second fish early life stage toxicity test, now with the next generation (F_{II}), in accordance with OECD guideline 210 (1).

According to the developmental characteristics of zebrafish the test was subdivided into the following periods:

Test period 1: Fish Early Life Stage Toxicity Test (F_I-generation - day 0-37)
Test period 2: Survival, growth, development and reproduction of F_I-generation (day 38 – day 134)
Test period 3: Fish Early Life Stage Toxicity Test (F_{II}-generation - day 135-174)

Materials and methods:

Table 1: Summary of test parameters and procedures of the full life cycle test with *D. rerio.*

Parameter		Measure, Setting or Condition
1.	Test substance:	NeemAzal
2.	Test type:	Full life cycle test with zebrafish (*Danio rerio*) (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae), 2 replicates with 100 fertilised eggs, each, at test start
3.	Test method:	flow-through system
4.	Nominal test concentrations:	0, 0.20, 0.63, 2.00, and 6.4 mg NeemAzal $\cdot l^{-1}$
5.	Test vessels:	
	day 1-14:	cages of (20 x 9 x 9 cm; length x depth x height), placed in 13.4 l glass aquaria (29 x 22 x 21 cm; length x depth x height)
	day 14-37:	13.4 l (total volume) glass aquaria (29 x 22 x 21 cm; length x depth x height). Volume of test solution = 12 l per vessel

Table 1: Summary of test parameters and procedures of the full life cycle test with *D. rerio (continued)*

Parameter		Measure, Setting or Condition
	from day 37 on:	29 l (total volume) glass aquaria (40 x 27 x 27 cm; length x depth x height). Volume of test solution = 25 l per vessel
	day 135-155 (F_{II}-generation)	cages of (20 x 9 x 9 cm; length x depth x height), placed in 13.4 l glass aquaria (29 x 22 x 21 cm; length x depth x height)
	day 155-174	13.4 l (total volume) glass aquaria (29 x 22 x 21 cm; length x depth x height). Volume of test solution = 12 l per vessel
6.	Dilution water:	purified drinking water
7.	Feeding:	
	F_I-generation:	
	from day 6 on:	breeding food (Tetra Werke, Melle)
	from day 9 on:	breeding food and nauplia larvae of *Artemia salina*
	from day 25 on:	breeding food, nauplia larvae of *Artemia salina* and TetraMin dry food (Tetra Werke, Melle)
		from day 56 on: TetraMin dry food (Tetra Werke, Melle) and nauplia larvae of *Artemia salina*
	F_{II}-generation:	
		from day 141 on: breeding food (Tetra Werke, Melle)
		from day 144 on: breeding food and nauplia larvae of *Artemia salina*
		from day 160 on: breeding food, nauplia larvae of *Artemia salina* and TetraMin dry food (Tetra Werke, Melle)
8.	Photo period:	12 h / 12 h (light / dark)
9.	Temperature:	mean temperature ± SD = 26.2 °C $\pm$ 0.3 °C (min. - max.: 24.5 - 26.8 °C; n = 96)
10.	pH-value:	between 7.72 ± 0.26 and 7.76 ± 0.26 (n = 97, each).
11.	dissolved oxygen:	between 6.2 ± 0.7 and 6.5 ± 0.6 mg/l (n = 96); corresponding to mean oxygen saturation values of 82 % to 85 %
12.	NeemAzal concentration:	analytically determined at least once weekly for every test vessel (see table 2)

Results and Discussion

Test concentrations of NeemAzal. Analytical values (as mean measured values) have been determined for each concentration step at least once a week. The data (see table 2) correspond to about 77 analytical double determinations for each concentration step.

Table 2: Test concentrations of NeemAzal given in mg/l.

Test vessel:	**1/1; 1/2**	**2/1; 2/2**	**3/1; 3/2**	**4/1; 4/2**
	Nominal concentrations of NeemAzal (mg/l)			
	0.20	**0.63**	**2.00**	**6.40**
mean measured value	**0.19**	**0.51**	**1.66**	**5.11**
± SD[a] (%)	78	66	26	22
% of nominal concentration	**95**	**81**	**83**	**80**

[a]SD = standard deviation

The four test concentrations were checked at least once per week for the whole test period. The basis for the determination of the NeemAzal content was the analysis of AzadirachtinA. Especially on behalf of the alkaline pH the degradation of AzadirachtinA is very rapid. Since AzadirachtinB degrades less rapidly we could estimate the degree of degradation from the AzadirchtinB/AzadirchtinA-ratio. The results indicate that the degradation of AzadirachtinA is very considerable and increases with the duration of the test (growth of the fish). Thus a considerable amount of degradation products especially of AzadirachtinA is present throughout the test period in addition to the stated amounts of NeemAzal (see table 2).

The mean measured concentrations over the whole test period of 174 days were in good agreement with the aspired values (not less than 80 % of the nominal concentrations) for all of the test vessels (see table 2). Hence, nominal concentrations were taken as basis for the calculation of effect concentrations.

Temperature, pH-values and content of dissolved oxygen during the test period: Water temperature and the content of dissolved oxygen met the validity criteria of the OECD guideline 210 (Fish, Early Life Stage Toxicity Test) (1), that means the oxygen content was not lower than 60 % at any time during the test and the temperature did not differ by more than ± 1.5 °C between test vessels or between successive days and was within the temperature range specified for *Danio rerio*.

Test period 1: Fish Early Life Stage Toxicity Test (F_1-generation - day 0-37)

Hatching rates, survival rates and fish lengths: Concerning the data obtained the following explanations must be added to the results, to distinguish clearly between effects caused by technical problems during the first week of the test, and effects which might have been caused by the test substance:

Hatching rates after 3 days: The hatching rate after 3 days was determined by regarding and counting the hatched larvae directly in the cages which stayed in the test vessels (glass aquaria with a total volume of 13.4 l). At this stage of development, the larvae had not swum up, yet. They lay on the nylon gauze that built the bottom of the cages. For the hatching rate after 3 days no significant differences compared to the control could be found.

Hatching rates/survival rates after 6 days: After 6 days most of the hatched larvae had swum up. For this reason the cages were taken out of the test vessels to photograph the larvae and count them out later (photo/computer).

It was started with the controls, then the lowest test concentration, and so on, up to the highest nominal concentration of 6.4 mg NeemAzal/l. By taking out the first cages from the control vessels, it became clear that some of the larvae tried to escape through the nylon gauze and some of them already hang with their tails in the meshes of the gauze. The next cages were now taken out with extreme care.

So, we can state that the relatively low survival rates in the controls and in the lowest test concentrations as well as the differences between the replicates can be attributed to the described technical problems.

The photos which were taken could not be evaluated because there was not enough contrast between the young larvae and the nylon gauze of the cages.

Therefore the data for this day 6 are regarded as "estimated".

Survival rates after 14 and 37 days: Because of the technical problems described above, some of the larvae were not in their cages anymore, but already escaped into the test vessels. To avoid additional stress, the larvae were not caught out of the vessels to determine the survival rate after 14 days exactly, but the number of animals in the vessels was estimated.

The survival rate after 37 days was determined, exactly, by taking photographs of the fish which could now be evaluated accurately.

There were no significant differences in survival rates in any of the test groups compared to the controls after 37 days.

Fish lengths: As a result of the different densities of fish in the test vessels, the animals showed relatively high variations in body length. For example, the highest mean length (1.864 cm) was found in the vessel with the lowest

survival rate (vessel No. 1/2, nominal concentration = 0.2 mg NeemAzal/l, see Figure 1).

In none of the test groups significant differences in fish lengths could be found compared to the controls. However, the results indicate that a dependence of the fish length on the fish densities may exist (see Figure 2). This has to be taken into account in later studies.

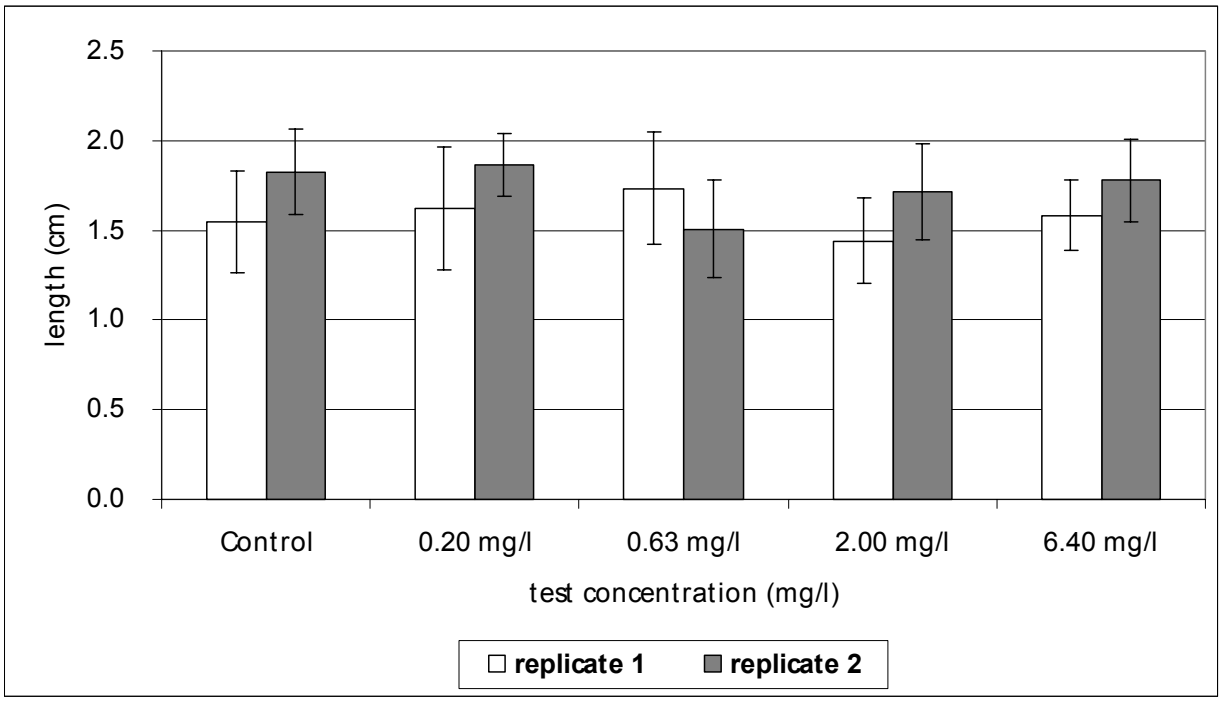

Fig. 1: Early Life Stage Test (F_1-generation) – mean fish lengths after 37 days.

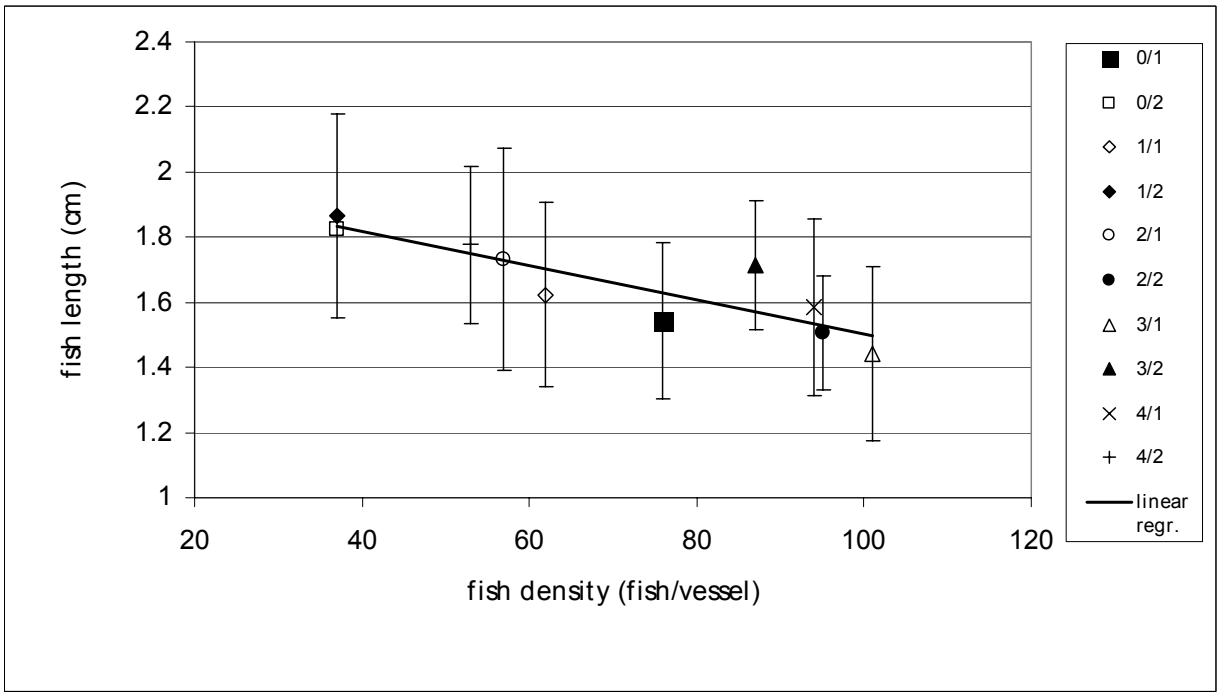

Fig. 2: F_1-generation - linear correlation between fish density and length at day 37 based on data listed in table 8. ($y = -0.0052*x + 2.0245$; $R^2 = 0.7696$).

Test period 2:

Survival, growth, development and reproduction of F_1-generation (day 38 - day 134)

Growth and survival of Fl-generation (figures 2 - 4)

Survival rates: Survival rates from day 50 to day 76 were high (96 % - 100%) for all test vessels. No differences compared to the control vessels were found in the test concentrations.

Fish lengths: The different fish densities which were explained and discussed in the last chapter, still influenced the growth of fish in the second test period.

Day 50 (see Figure 3): Comparing the mean values of fish lengths for each test concentration with the mean value for the control (n = 2), no significant differences (p level = 0.05) were found. Within the test concentrations with 0.63, 2.0 and 6.4 mg NeemAzal/l significant differences between the respective replicates were found. By testing single replicates of the test concentrations against single control replicates significant differences were found for the following replicates: 2/2 (0.63 mg NeemAzal/l), 3/1 (2.00 mg NeemAzal/l) and 4/1 (6.4 mg NeemAzal/l). Again, a direct correlation to fish density can be stated for these effects: The listed replicates were those with the highest number of fish (95, 101, and 94).

Day 76 (see Figure 4): Comparing the mean values of fish lengths for each test concentration with the mean value for the control (n = 2), no significant differences (p level = 0.05) were found. Within the test concentrations with 6.4 mg NeemAzal/l a significant difference between the replicates was found. By testing single replicates of the test concentrations against single control replicates significant differences were found for the following replicates: 2/2 (0.63 mg NeemAzal/l), 3/1 and 3/2 (2.00 mg NeemAzal/l, each), and 4/1 (6.4 mg NeemAzal/l). Again, a direct correlation to fish density can be stated for these effects: The listed replicates were those with the highest number of fish (95, 101, 87 and 94).

'Pseudo' specific growth rates based on fish length (day 50 - day 76)

According to the effects of fish density on fish length, the different fish densities affected the growth of fish in a way, that those test vessels with the shortest fish (see Figure 3 and 4) (and the highest densities until day 50) showed the highest 'pseudo' specific growth rates (see Figure 5).

Comparing the mean values of fish lengths for each test concentration with the mean value for the control (n = 2), no significant differences (p level = 0.05) were found. Within the control as well as within the test concentrations with 0.63 and 2.0 mg NeemAzal/l significant differences between the respective replicates were found (see table 11, p-value (w)). By testing single replicates of the test concentrations against control replicate 0/1 significant differences

were found for the following replicates: 0/2 (control), 1/2 (0.2 mg NeemAzal/l), 2/1 (0.63 mg NeemAzal/l), 3/1 and 3/2 (2.00 mg NeemAzal/l, each). Comparison of single replicates with control replicate 0/2 showed significant differences with replicates 0/1 (control), 1/1 (0.2 mg NeemAzal/l), 2/2 (0.63 mg NeemAzal/l), 3/1 (2.00 mg NeemAzal/l) and 4/1 and 4/2 (6.4 mg NeemAzal/l, each).

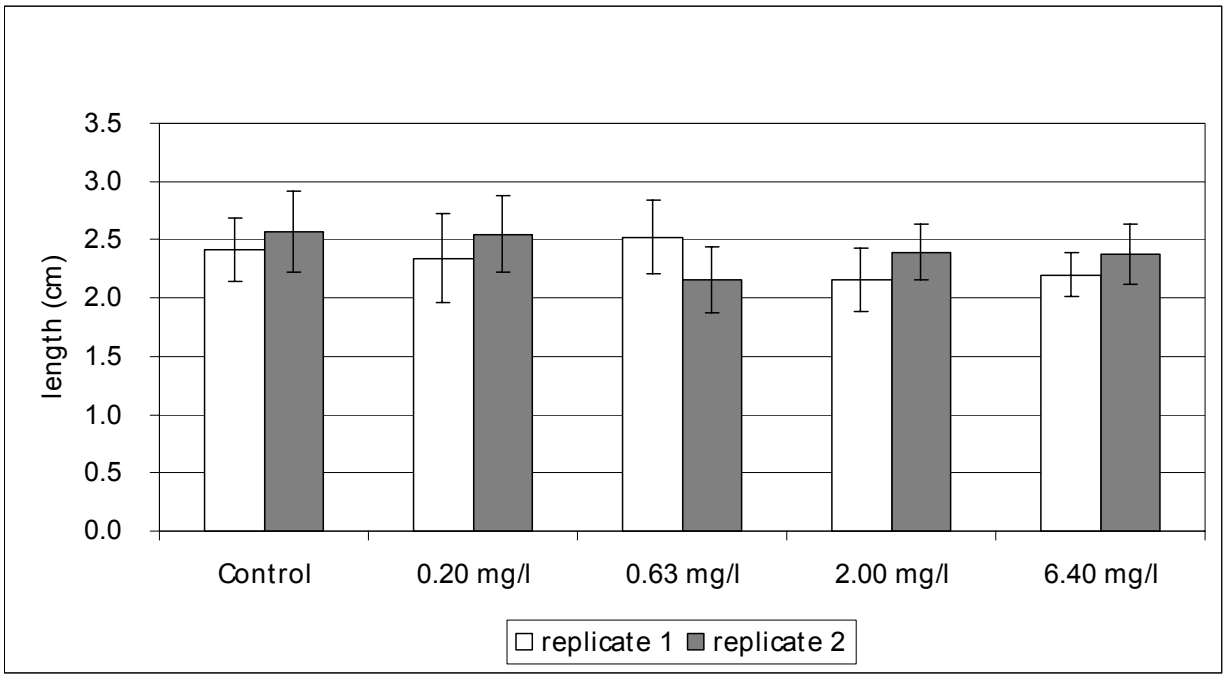

Fig. 3: F_1-generation – fish lengths (cm) at day 50 of the test.

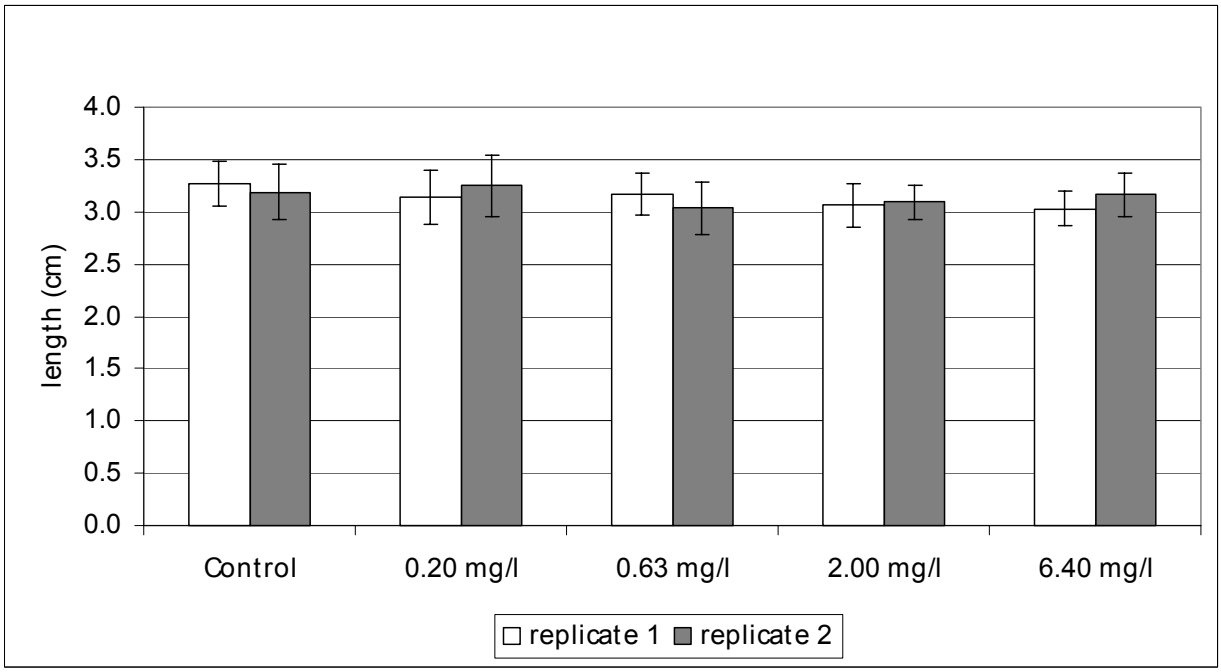

Fig. 4: F_1-generation – fish lengths (cm) at day 76 of the test.

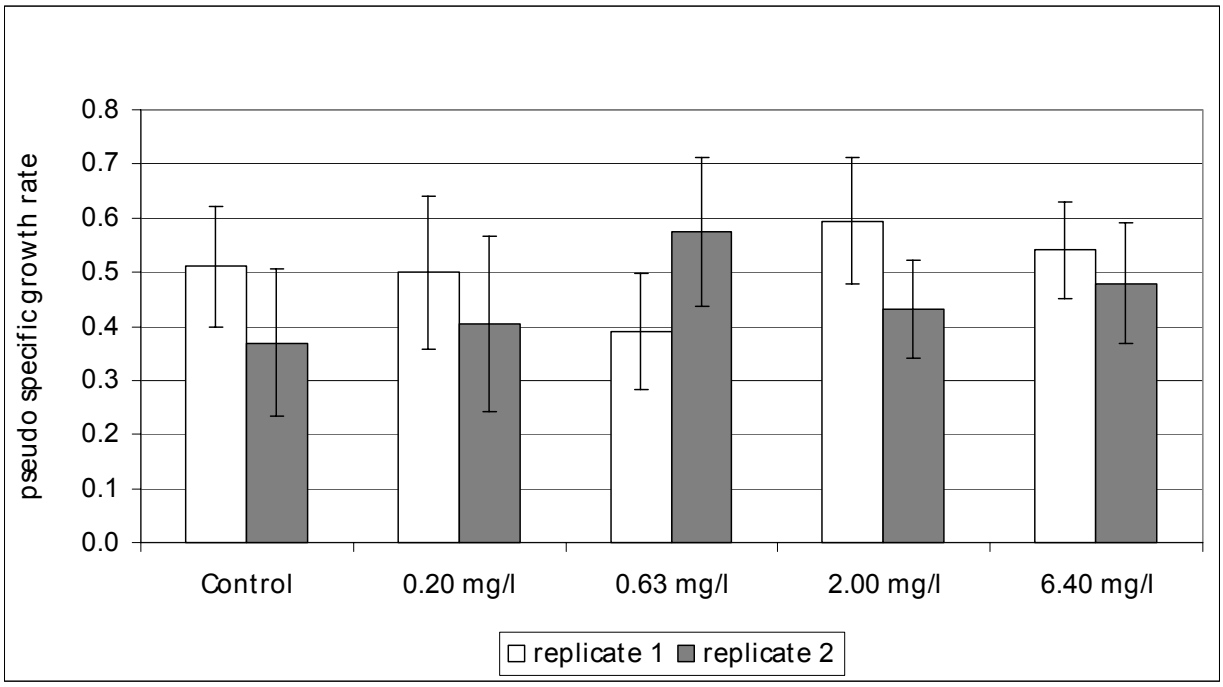

Fig. 5: F_1-generation – 'pseudo' specific growth rates from day 50 to day 76 (mean values standard deviation) based on fish length (cm) at day 76 of the test.

Fish weight: At day 50 and day 76 fish were weighed in total for each test vessel and the average weight per fish was calculated. Neither for the fish weight values at day 50 and day 76 nor for the increase of weight, expressed as the tank-average specific growth rate significant differences between the test concentrations and the controls could be found. However, the same tendencies as for the correlation between fish densities and fish length (see above) can be stated for fish weight and increase of fish weight between day 50 and day 76: low fish density led to relatively high fish weight and low increase of weight and vice versa.

Reproduction of F_1-generation

Time of reaching sexual maturity: According to (2) zebrafish can be expected to reach sexual maturity with an age of about 90 days. Mating behaviour was observed at day 78. Test fish showed the same behaviour in the control groups as well as in the groups which were exposed to the test substance. Eggs were found in all spawning-trays at day 78. Therefore, no effect of the test substance on the time of first egg production could be detected up to the highest test concentration of 6.4 mg NeemAzal/l.

Egg production and fertilisation rate: Comparing the mean values of total number of eggs per test vessel and day for each test concentration with the mean value of the control (n = 2), no significant differences (p level = 0.05) were found. Within the control and all of the test concentrations no significant differences between the respective replicates were foun. By testing single

replicates of the test concentrations against single control replicates significant differences were found for the replicates 2/1 and 2/2 with 0.63 mg NeemAzal/l. As no differences within the controls or within the test concentrations were detected, the data of the respective replicates were united to one set of data and statistically evaluated. The total number of eggs per test vessel and day was significantly reduced in 0.63 mg NeemAzal/l. No effects of the test substance on the fertilisation rates were observed.

The mean number of fertilised eggs per female and day was statistically evaluated following the same procedure as used for the total number of eggs per test vessel (see above). A significant reduction of number of fertilised eggs per female and day was found in 0.63 mg NeemAzal/l (see Figure 6 and 7).

In contrast to the toxicological model of concentration-response-relationship the observed difference did not increase with increasing concentrations of the test substance NeemAzal.

So the mean number (± standard deviation) of fertilised eggs per female and day was 41 ± 8 in the control and 41 ± 2 in 2.00 mg NeemAzal/l. In the highest test concentration with 6.4 mg NeemAzal/l the respective value was even higher with 49 ± 4 fertilised eggs per female and day.

The results of figure 6 and 7 clearly demonstrate that:

- the replicates of the control group and the test concentration groups ran parallel,
- from the beginning of data recording, the total number of eggs was lower and increased more slowly in 0.63 mg NeemAzal/l than in the controls and more slowly than in all other test concentrations, including the two higher test concentrations (2.00 and 6.4 mg NeemAzal/l) over the period of 21 days (figure 6),
- compared to the control, the number of fertilised eggs per female and day was significantly lower in 0.63 mg NeemAzal/l, only.

Sex ratio of F_I-generation: The sex ratio of F_I-generation at day 84 is shown in figure 8. Variations between the different concentrations are not statistically significant.

Test period 3: Fish Early Life Stage Toxicity Test (F_{II}-generation - day 135-174)

Hatching rate, survival rate, fish length and total fish weight

Hatching rates, survival rates: No significant differences in the hatching rates and survival rates between the control group and the groups treated with NeemAzal occurred during the 39 days of the FELS (fish early life stage toxicity test) with F_{II}-generation.

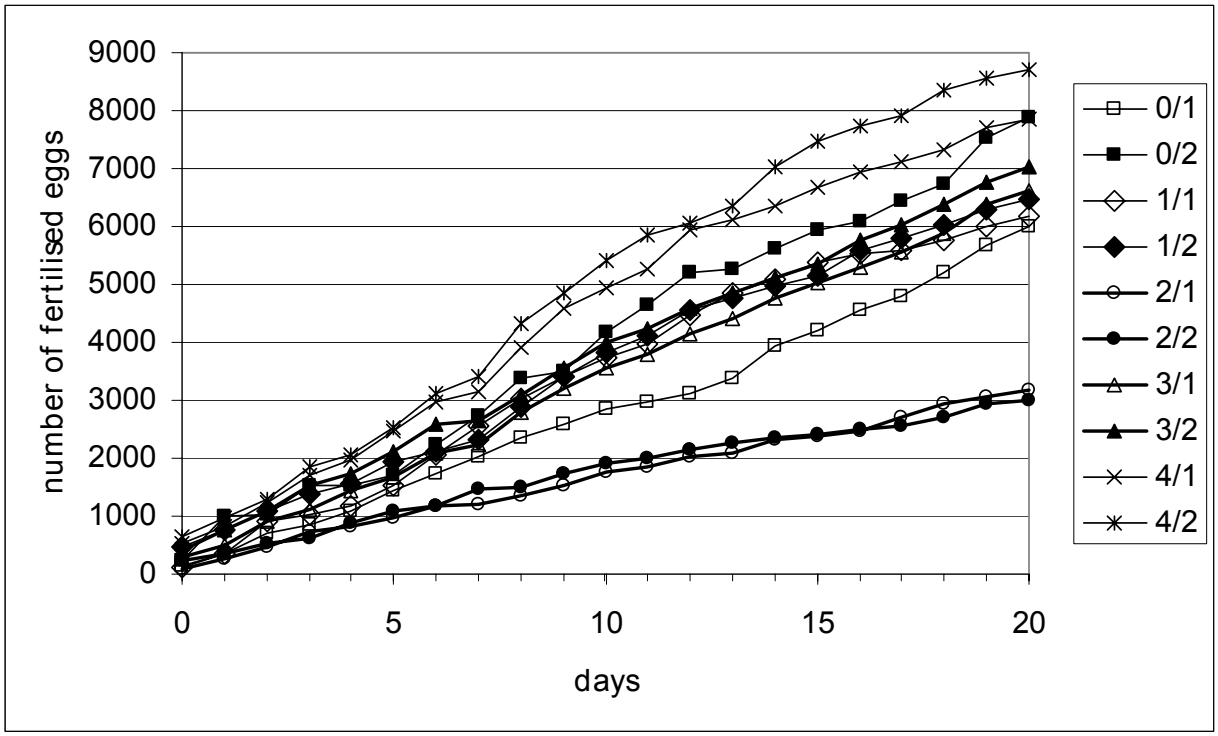

Fig. 6: F_1-generation – cumulative number of fertilised eggs per vessel, determined over a period of 20 days (n = 21). Legend gives the number of test vessels. For nominal test concentrations see table 2.

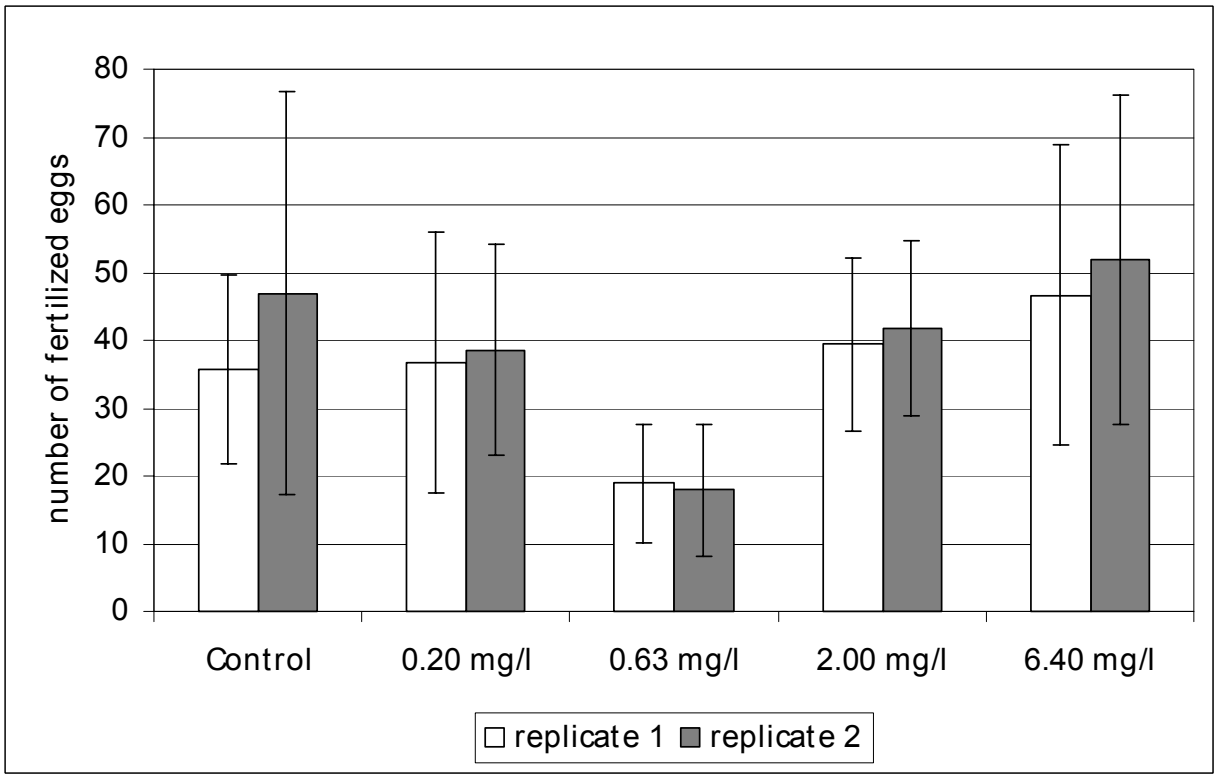

Fig. 7: Mean number of fertilised eggs (n) per female and day. Standard deviation indicated as error bars, n = 21.

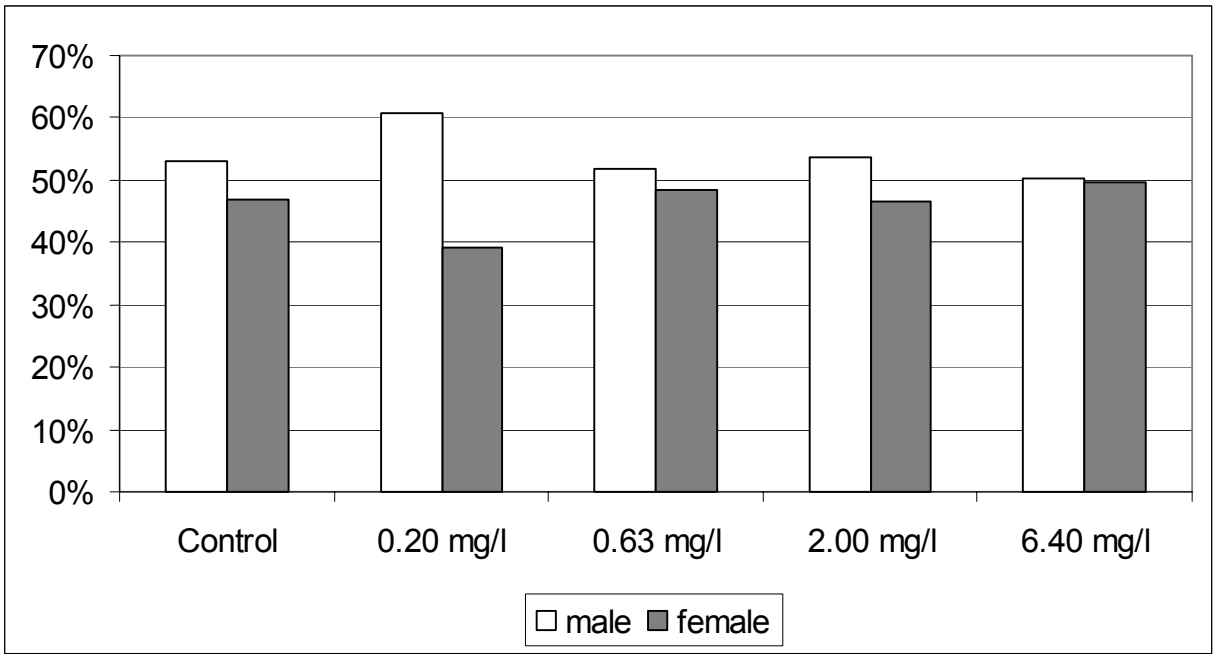

Fig. 8: Sex ratio of F_I-generation in % of total number of fish. Fish which were not used for investigations on reproduction (from day 84 on) are included.

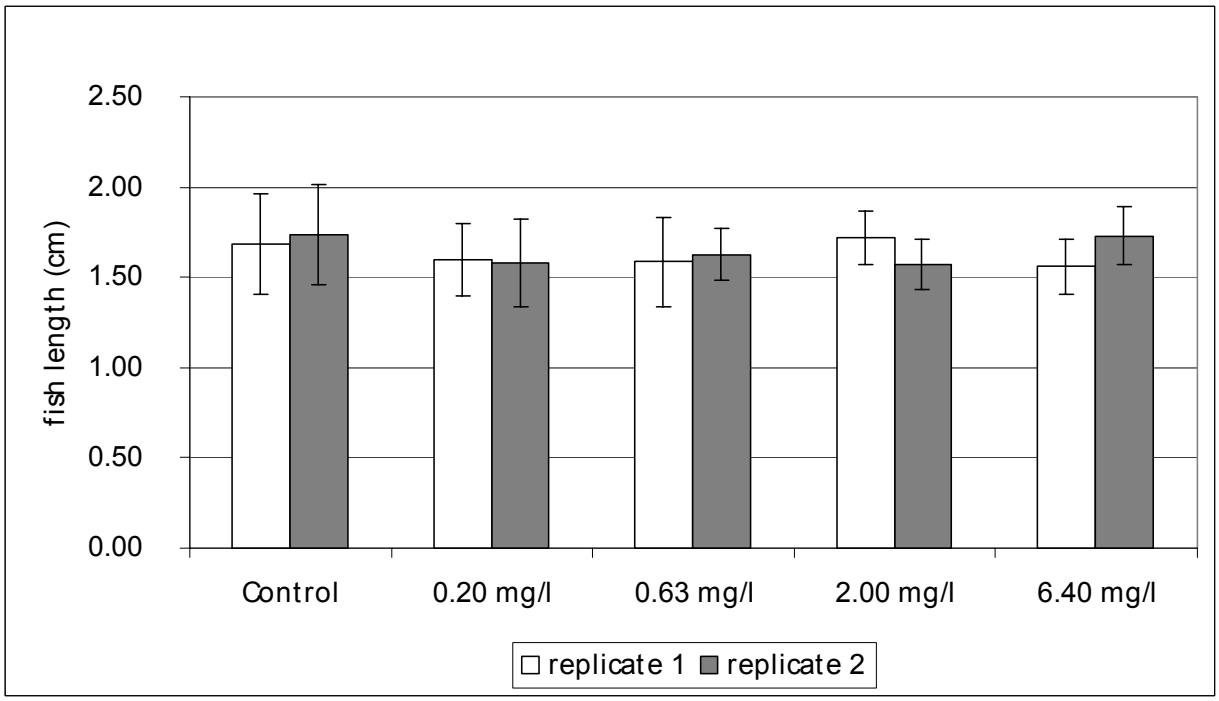

Fig. 9: F_{II}-generation – fish lengths (cm) at day 39 of the second early life stage test (= day 174 of the full life cycle test).

Fish length: Comparing the mean values of fish lengths (day 39 of the F_{II}-generation) for each test concentration with the mean value for the control (n = 2) (see Figure 9), no significant differences (p level = 0.05) were found. Within the test concentrations with 2.0 and 6.4 mg NeemAzal/l significant differences between the respective replicates were found.

Fish weight: At day 39 of the second fish early life stage toxicity test fish were weighed in total for each test vessel and the average weight per fish was calculated. No significant differences between the test concentrations and the controls could be found (see Figure 10).

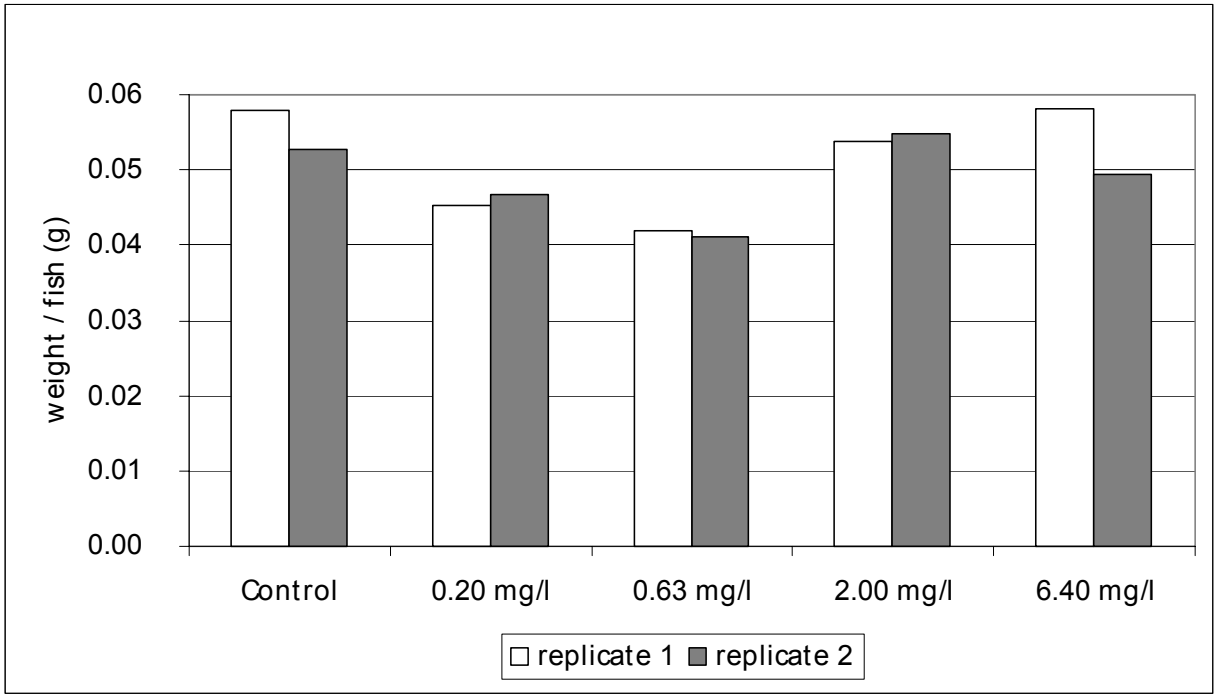

Fig. 8: F_{II}-generation – weight per fish (g) at day 39 of the second early life stage test (= day 174 of the full life cycle test).

The main results of the zebrafish - full life cycle test with NeemAzal as test substance are summarized in table 3.

Table 3: Endpoints of the full life cycle test with NeemAzal. Differences between groups (2 replicates, each) which were exposed to the test substance and the control group, with "n.s." = not significant and
"*" = significant ($p \leq 0.05$, for both replicates checked against both replicates of the control).

Test Period	Endpoint	Nominal concentrations of NeemAzal (mg/l) 0.20	0.63	2.00	6.40
1	**Hatching rate**, day 3	n.s.	n.s.	n.s.	n.s.
	Survival rate, day 37	n.s.	n.s.	n.s.	n.s.
	Fish lengths, day 37	n.s.	n.s.	n.s.	n.s.
	Fish weight, day 37	n.s.	n.s.	n.s.	n.s.
2	**Survival rate**, day 50-76	n.s.	n.s.	n.s.	n.s.
	Fish lengths:				
	- at day 50	n.s.	n.s.	n.s.	n.s.
	- at day 76	n.s.	n.s.	n.s.	n.s.
	Fish weight:				
	- at day 50	n.s.	n.s.	n.s.	n.s.
	- at day 76	n.s.	n.s.	n.s.	n.s.
	Growth:				
	- 'pseudo' specific growth rate (based on fish length)	n.s.	n.s.	n.s.	n.s.
	- tank-average growth rate (based on fish weight)	n.s.	n.s.	n.s.	n.s.
	Reproduction:				
	- time of reaching sexual maturity				
	- egg production	n.s.	n.s.	n.s.	n.s.
	- fertilisation rate	n.s.	*	n.s.	n.s.
		n.s.	n.s.	n.s.	n.s.
3	**Hatching rate:**				
	- at day 3	n.s.	n.s.	n.s.	n.s.
	- at day 4	n.s.	n.s.	n.s.	n.s.
	Survival rate:				
	- at day 6	n.s.	n.s.	n.s.	n.s.
	- at day 14	n.s.	n.s.	n.s.	n.s.
	- at day 27	n.s.	n.s.	n.s.	n.s.
	- at day 39	n.s.	n.s.	n.s.	n.s.
	Fish lengths, day 39	n.s.	n.s.	n.s.	n.s.
	Fish weight, day 39	n.s.	n.s.	n.s.	n.s.

References

(1) OECD Guideline for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, No. 210 "Fish, Early Life Stage Toxicity Test". Adopted July 17th, 1992, Paris. 1993.

(2) Nagel, R (1998) Der Vollständige Life Cycle Test (Complete Life Cycle Test, CLC-Test) mit dem Zebrabärbling (*Danio rerio,* vormals *Brachydanio rerio*), Entwurf. UBA-Texte, in press.

QUANTIFICATION OF AZADIRACHTIN IN NEEM PRODUCTS AND IN DIFFERENT COMPARTMENTS AFTER NEEM-TREATMENT

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Quality control of Neem material:

Azadirachtin A (Fig. 1) is established as the leading compound for analytical purposes of Neem materials which are usually seed kernels, oil and formulations.

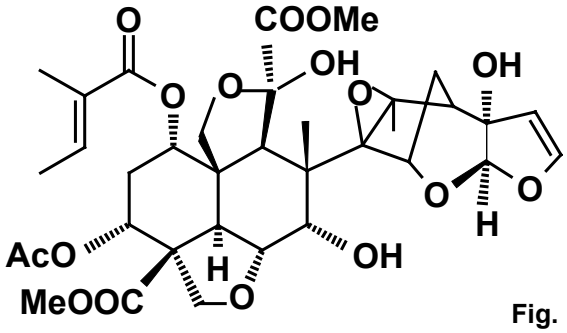

Fig. 1. Azadirachtin A

Analytical tools for quantification of Azadirachtin A may be:

TLC	Thin Layer Chromatography - simple method, about 100 years old, good results for qualification
SFC	Supercritical Fluid Chromatography - mobile Phase: gases (i.e. CO_2) in supercritical conditions
HPLC	High Performance Liquid Chromatography
HPLC / MS	HPLC / Mass Spectroscopy - technical equipment and maintenance is very expensive

The HPLC has become the routine method for analysis of Azadirachtin A, because it is a reproducible and robust method which gives fast results and has the advantage of low detection limits. The setup for HPLC analysis of Azadirachtin A is as follows:

UV detection:	at 214 nm (absorption maximum of AzA: 220 nm)
Column:	RP material, C18 or C12, particle size: 3 – 5 µm
Eluents:	CH_3CN or CH_3OH and H_2O

A gradient system is preferable but it is also possible to use an isocratic system.

In 1996 a standard method for sample preparation and analysis (CIPAC method no. 4042[1]: “High performance liquid chromatographic method for the analysis of Azadirachtin in Neem kernels, Neem oil and formulated products”) was developed and established. The aim was to create a simple and standardized method because results of ring trials showed varying results in terms of determination of Azadirachtin A contents. The deviations of the Azadirachtin A content in similar Neem seed kernel material varied from 1,56 to 7,47 mg Azadirachtin A per g seeds. The ring trials were carried out by laboratories in India, Germany, Thailand, the UK and Kenya.

For comparison of the analytical results the equipment, chemicals and standard preparations should be as similar as possible in every laboratory. Other aspects should also be followed:

- At least two independent analysis solutions from each sample should be analysed.
- Each sample has to be analysed in duplicate.
- The Azadirachtin A content should be measured within the calibrated concentration range.
- The calculation of the Azadirachtin A content in Neem kernels is based on dried material.[2]

Information about the quality of Neem kernels is very important, because they are the starting material for all Neem products used for pest control. Table 1 shows results of different samples of Neem kernels in view of the Azadirachtin A and the corresponding moisture content. To use the Neem kernels as starting material for e.g. NeemAzal they should at least have a Azadirachtin A content of 3 mg/g.

The moisture content is also an important factor in respect of the stability of Azadirachtin A in Neem kernels. High moisture contents in the kernels or storage of the kernels under humid conditions initiate a rapid degradation of Azadirachtin A[3]. To store the kernels and maintain the original Azadirachtin A content it is indispensable to reduce the moisture in kernels below 8%.

Table 1 Azadirachtin A and moisture content in different Neem seed kernels

Neem kernels	AzA content [mg/g]	moisture content [%]
Ghana (different regiones)	5,5	4,8
	4,2	4,9
	4,9	5,6
	5,4	5,1
Senegal	3,2	5,6
Columbia	4,8	6,7
Malawi	2,7	7,5
Madagaskar	2,3	4,2
Iran	1,8	4,7
Brazil	3,7	42,5!

The Brazilian Neem kernels show a very high moisture content. This is due to the fact that the kernels were fresh and haven't been dried. Further experiments showed that Neem kernels from Brazil which were dried properly had a moisture content of approximately 7,5%.

Residue analysis of Azadirachtin A in different matrices

Residue analysis is a necessary and important tool for the evaluation of the mode of action (efficacy), of the risk estimation in respect of consumer protection (residues in foodstuff) and for the evaluation of possible hazards in environmental compartments, e.g. water (ecotoxicology).

Residue analysis is implemented by the authorities. In most countries results concerning residue analysis are ONLY accepted by the authorities if they are prepared according to GLP.

GLP – Good Laboratory Praxis - is a quality system concerned with the organisational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported. GLP ensures the generation of high quality and reliable test data in the framework of harmonising testing procedures for the mutual acceptance of data.

To achive a GLP-certificate, the test facility is inspected by the federal authorities and inspections on a regular basis are inevitable for maintenance of the certificate.

The implemented principles of GLP include:

- A quality assurance program to ensure that studies are performed in compliance with the principles of GLP
- Personnel: appropriate qualified persons for the relevant positions
  sufficient number of persons to carry out the necessary tasks
- SOPs (Standard Operating Procedures) – instructions relevant to all activities being performed in the test facility:
  - Organisation and personnel
  - Quality assurance
  - Facilities (laboratories, rooms)
  - Apparatus and materials
  - Evaluation of results
  - Test systems
  - Test and reference items (e.g. standard solutions of AzA)
  - Conduct of the study (e.g. study plan)
  - Recording (raw data, reports)
  - Archiving

Residue studies:

A prerequisite for residue analysis is a development of a suitable method, which is normally the extraction of Azadirachtin A from a matrix with subsequent purification and HPLC determination of the analyte. The development is followed by the validation of the method. In the context of the validation several parameters like limit of detection, limit of quantification, recovery rate, accuracy and precision, specificity and selectivity of the method are determined.

After a successful validation residue studies may be carried out. For acceptance by the authorities GLP is compulsory for the whole study (even for field parts).

Residue studies in water are important for the control of the concentrations of NeemAzal-T/S and NeemAzal in tests of toxicological and ecotoxicological relevance, e.g. studies on fish, daphnia and algae. It is important to know that the half-life time of Azadirachtin A in water is dependent on temperature and pH, see table 2.

Table 2 Stability of Azadirachtin A in water

pH	Temperature [°C]	half-life time [d]
4	20	49,9
	30	10,7
	40	1,2
7	20	19,5
8	20	4,4

Method for residue analysis in water

An extraction procedure to isolate Azadirachtin A out of water samples is not necessary. Depending on the concentration of Azadirachtin A in the sample they have to be concentrated. Because of the rapid degradation of Azadirachtin A in water at higher temperatures it is advisable to avoid evaporation of the surplus water and use instead solid phase extraction methods. A limit of determination of 0,1 µg AzA per litre can be obtained.

Residue analysis in soil is important for monitoring and controlling the concentrations of NeemAzal and NeemAzal-T/S in tests of ecotoxicological and environmental relevance, like adsorption/desorption studies, leaching activity, degradation in soil and side effects on soil micro flora (earthworms).

Method for residue analysis in soil

Approx. 50 g soil has to be extracted with 50 ml CH_3OH followed by filtration and washing. Afterwards a solid phase extraction for purification has to be performed. It may be necessary to make use of different solid phase sorbents to obtain a sufficient purificated extract. The limit of determination is 10 µg AzA per kg soil.

Method for residue analysis in plant material

Residue analysis in plant material is necessary for the evaluation of MRL values (Maximum Residue Levels) and ADI values (Acceptable Daily Intake) by the authorities. These values are important for the evaluation of waiting periods after the last treatment of plant material with NeemAzal-T/S. The aim of this work is the protection of the consumer.

First step is an extraction procedure where the plant material has to be homogenized thoroughly. The amount of plant material and the choice of the solvent is dependet on the plant matrix. At least two solid phase extractions are necessary (polar and nonpolar sorbents).

A problem with the analysis of AzA in plant material arises and this is the absorption maximum of Azadirachtin A (220 nm) where many plant matrix components interfere with Azadirachtin A in the HPLC chromatogram, which results in a costly separation of the extracts.

It is not possible to present a general residue method for extraction of all plants, which have a good potential with respect to registration. That means that nearly every plant needs its own development and validation of a method which is time consuming and expensive.

Results of residue studies have shown that there is a possiblily to divide plant material in two groups:

- leafy vegetables (large surface to mass ratio, e.g. lettuce, spinach, etc.)
- fruity vegetables and fruits (small surface to mass ratio, e.g. tomatoes, apples, etc.)

To predict possible results we propose a general concept for the estimation of Azadirachtin A residues on/in plants and make following assuptions:

Yield of crop	10 tons per hectare
Amount of AzA	3 litres NeemAzal-T/S per hectare per treatment, i.e. a maximum of 30 g Azadirachtin A per hectare
Assumption	100% of the active ingredient reach the plants
Residues on plants	3 mg Azadirachtin A per kg (= 3ppm)

A comparison of the decrease of Azadirachtin A on/in tomato leaves (representative for leafy vegetables) and tomatoes (representative for fruity vegetables) is presented in the following diagrams (Fig. 2 and Fig. 3).

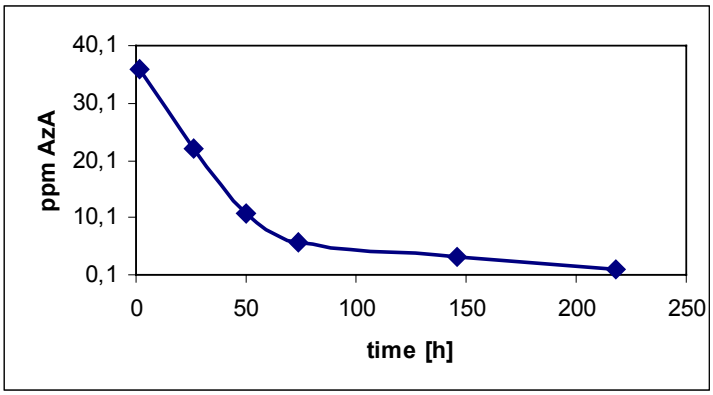

Fig. 2 Azadirachtin A on/in tomato leaves after treatment with the tenfold recommended concentration (5% NeemAzal-T/S); $t_{1/2}$ is approx. 1 day.

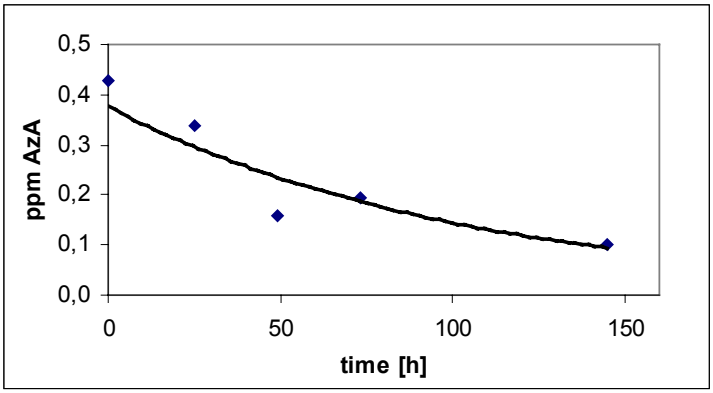

Fig. 3 Azadirachtin A on/in tomato after treatment with the tenfold recommended concentration (5% NeemAzal-T/S); $t_{1/2}$ is approx. 3 days.

Aside from the different half-life-times in tomato leaves and tomatoes the concentration of Azadirachtin A directly after treatment is different as well. Other residue studies show the same tendency - the leafy vegetables show a higher initial concentration of Azadirachtin A (approx. 3 mg Azadirachtin A per kg) whereas the fruity vegetables and fruits show an initial concentration of less than 0,1 mg Azadirachtin A per kg.

The decrease of Azadirachtin A in/on plants is quite rapid. HPLC chromatograms of tomatoes, which were treated, with the 10fold-recommended concentration of NeemAzal-T/S are shown in Fig. 4.

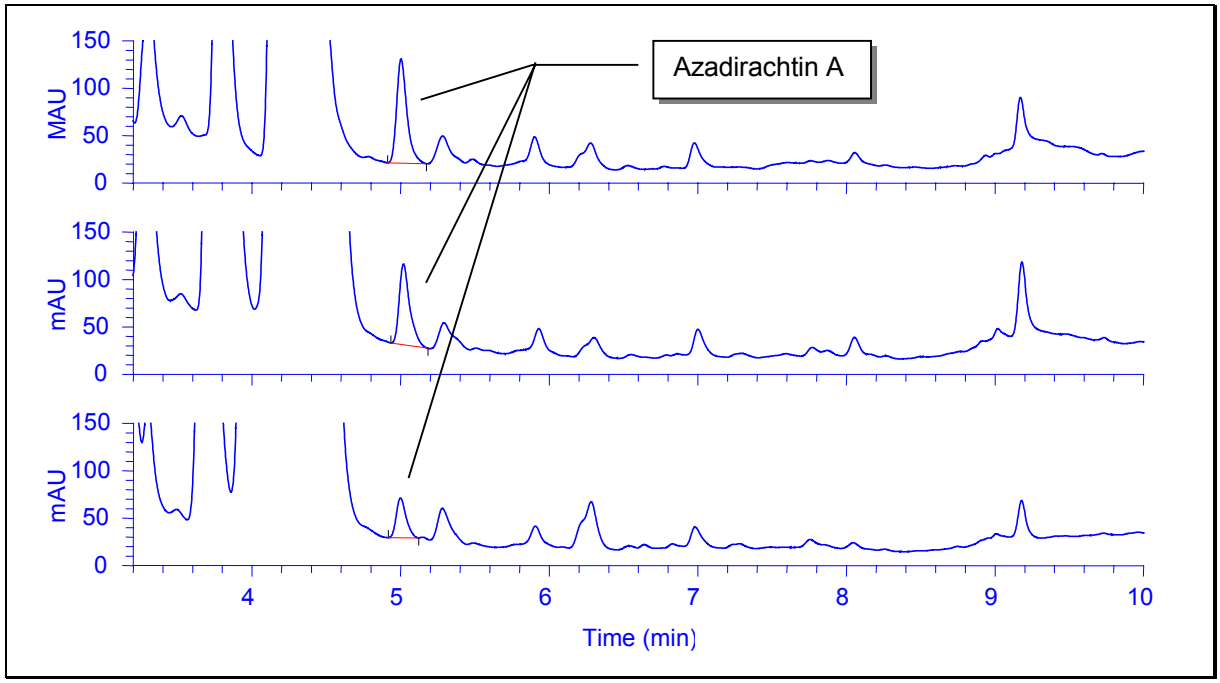

Fig. 4 chromatograms of tomates which were treated with NeemAzal-T/S
upper chromatogram: day of treatment = 0,43 ppm AzA
middle chromatogram: 1 day after treatment = 0,34 ppm AzA
lower chromatogram: 2 days after treatment = 0,16 ppm AzA

Those results help us to propose waiting periods for the estimation of residues in plant material. On basis of the dietay regulations (Diätverordnung) which is the strictest limit of the German authorities regarding residues in food (it demands that less than 0,01 mg residue per kg is existing) we propose waiting periods of 8-9 days after treatment for leafy vegetabels and 9-12 days for fruity vegetables and fruits, respectively.

Only food sold under the indication “dietary food” has to follow the regulation of 0,01 mg/kg.

The MRLs and the waiting periods for other products, like organically grown food, will be evaluated by the authorities and the limit is expected to rise up to a 100 times compared to the “Diätverordnung” and consequently no waiting period is expected.

We expect an acceptable daily intake (ADI) for Azadirachtin A of 0,1 to 1 mg/kg food.

[1] CIPAC no 4042; Collaborative Study for the Determination of Azadirachtin A in Neem Seed Kernels, Aqueous Formulation and oil, Dr. Josef Brodesser, GTZ Pesticide Service Project, Germany, June 1998

[2] International Rules for Seed Testing Rules 1996, International Seed Testing Association (1996), Seed Sci. & Technol. 24, Supplement

[3] K. Ermel, E. Pahlich and H. Schmutterer, Natural pesticides form the Neem tree and other tropical plants, 1986, 171-184

POTENCIAL DO USO, PRODUÇÃO DE EXTRATOS DE PLANTAS BRASILEIRAS E DESENVOLVIMENTO DE PRODUTOS PARA O CONTROLE DE PRAGAS E ECTOPARASITOS EM ANIMAIS E SERES HUMANOS: PLANTAS INSETICIDAS.

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O crescimento contínuo da população do mundo, associado à grande competição entre o homem e insetos pela alimentação, a transmissão de doenças e infecções hospitalares tendo como vetores os insetos associados á resistência a inseticidas existentes por alguns insetos, levam á necessidade de uma contínua busca de novos inseticidas.

O uso de inseticidas representa gastos na ordem de bilhões de dólares em um esforço de controlar insetos. Os inseticidas mais usados têm sido os piretróides e os organofosforados seguido dos organoclorados. Esses inseticidas apresentam um amplo espectro de ação e devido a isso exterminam os insetos nocivos, bem como os que são considerados benéficos ao homem. Além disso, eles podem adquirir resistência a esses inseticidas, de tal forma que sempre haverá a necessidade de aplicação em quantidades cada vez maiores para um controle efetivo, causando danos ecológicos ao meio ambiente (Mariconi, 1981).

A necessidade de métodos eficazes e cada vez mais seguros no controle de insetos tem estimulado a busca de novos inseticidas em plantas. Um exemplo de busca bem sucedida está no uso de piretróides, extraídos das flores de crisântemos (espécies do gênero *Chrysanthemum* - Asteraceae). A grande vantagem do uso de piretróides consiste na baixa toxicidade dos mesmos para mamíferos. Isso fez impulsionar a utilização e as pesquisas até nossos dias.

As plantas têm sido uma importante fonte de substâncias químicas ativas contra insetos, tais como a nicotina, nornicotina e anabasina (alcalóides), os piretróides (piretrina e aletrina) e os rotenóides. (Crosby, 1971). Porém o seu uso direto ou de seus extratos brutos se limita a aplicações domésticas.

Produtos naturais inseticidas foram muito utilizados até a década de 40. Á partir dessa data, diversos biocidas sintéticos surgiram como subproduto das pesquisas durante a Segunda Grande Guerra, tais como o DDT, BHC, Aldrin, Dieldrin e Clordano, sendo esses muito mais potentes e também com menor especificidade de ação no combate às pragas agrícolas que os naturais, e assim substituíram completamente os inseticidas naturais.

O retorno à busca de novos inseticidas naturais, surgiu devido à capacidade aleatória de adaptação da natureza em relação ao uso indiscriminado de inseticidas sintéticos pelo homem e, o fato do homem ter começado a refletir um pouco mais sobre a relação inseto-planta. Dessa forma, alertados por trabalhos como o de Carson - Livro Silent Spring (1962) passou a respeitar um pouco mais os mecanismos naturais de adaptação.

Para o uso de inseticidas botânicos, diversos aspectos devem ser levados em consideração: extração, conservação dos extratos, dosagem eficiente, estabilidade, toxicidade e custo. Todos esses aspectos são compreendidos quando identificadas as principais substâncias contidas neste inseticida.

As principais plantas das quais são obtidas substâncias com atividades inseticidas pertencem aos gêneros *Nicotiana* (Solanaceae), produtoras de nicotina e nornicotina; *Derris, Lonchocarpus, Tephrosia e Mundulea* (Leguminosae), produtoras de rotenóides, *Chrysanthemum* (Asteraceae), produtoras de piretrinas e *Azadirachta* (Meliaceae), produtoras de azadiractina. Dentre as principais formas de controle inseticida efetuado por plantas pode-se destacar a ação fagoinibidora do extrato da planta, a qual inibe a alimentação, mas não mata o inseto diretamente e os mesmos são levados à morte por fome. Uma substância pode apresentar ação deterrente, a qual impede a alimentação do inseto.

Dentre as plantas que apresentam atividades inseticidas pode-se destacar a classe dos rotenóides (rotenona e tefrosina) que apresentam atividade fagoinibidora, os piretróides (piretrinas, jasmolinas e cinerinas) extraídas das flores de crisântemos, os alcalóides (nicotina e nornicotina, metilcaconitina) que inibem a os receptores da acetilcolinesterase, os terpenóides (tenulina, angulatina A) e principalmente a azadiractina (isolada da planta *Azadirachta indica*, conhecida como neen) que possui ação interferente no funcionamento das glândulas endócrinas que controlam a metamorfose dos insetos, impedindo o desenvolvimento da ecdise, apresentando, ainda, atividade fagoinibidora. Várias são as estratégias capazes de determinar a atividade de produtos de origem natural contra insetos e o seu isolamento. De uma maneira geral, inicia-se com extratos brutos de plantas preparados com diversos solventes (hexano, diclorometano, acetato de etila, metanol e água). Em seguida, os extratos ativos são fracionados através dos métodos cromatográficos existentes e as frações obtidas são retestadas, repetindo-se o processo até a obtenção do(s) composto(s) ativo(s). Em seguida escolhe-se o bioensaio mais apropriado para determinar a atividade inseticida, que depende dos hábitos dos insetos com o qual se deseja fazer o ensaio. Para determinar a capacidade de certas substâncias em repelir insetos, utiliza-se principalmente o tratamento das folhas das plantas, pois se sabe que as mesmas são comidas por larvas de insetos.

Compreende-se hoje que o combate a uma determinada praga não pode ser feito de uma maneira unilateral. O controle de insetos deve ser integrado, com o uso de

feromônio (associados a inseticidas), inimigos naturais (através de um controle biológico), produtos naturais, deterrentes que induzem mutações para alcançar a esterilidade nas próximas gerações de insetos e outras estratégias que busquem não a erradicação do inseto-alvo, mas sim um equilíbrio ao já desequilibrado ecossistema. O uso da quimiossistemática pode auxiliar na obtenção de novos inseticidas naturais, como a azadiractina, que pode ser considerada como o mais recente inseticida natural.

Dessa maneira, a grande variedade de substâncias presentes na flora continua sendo um enorme atrativo na área de controle de insetos e pragas, principalmente levando-se em consideração que apenas uma pequena parcela dessas plantas foi investigada com tal objetivo.

POTENTIAL OF THE USE, PRODUCTION OF EXTRACTS OF BRAZILIAN PLANTS AND DEVELOPMENT OF PRODUCTS FOR THE CONTROL OF PLAGUES AND ECTOPARASITOS IN ANIMALS AND HUMAN BEINGS: INSECTICIDE PLANTS.

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The continuous growth of the population of the world, associated to the great competition between the man and insects for the feeding, the transmission of diseases and infections hospitalares tends as vectors the insects associated a resistance to existent insecticides for some insects, they take a need of a continuous search of new insecticides.

The use of insecticides represents expenses in the order of billion of dollars in an effort of controlling insects. The used insecticides have been the piretroids and the organophosphors followed by the organochlords. Those insecticides present a wide action spectrum and due to that they exterminate the noxious insects, as well as the ones that are considered beneficial to the man. Besides, they can acquire resistance to those insecticides, in such a way that there will always be the application need in amounts every larger time for an effective control, causing ecological damages to the environment (Mariconi, 1981).

The need of effective and more and more safe methods in the control of insects has been stimulating the search of new insecticides in plants. A search example well happened it is in the piretróides use, extracted of the flowers of chrysanthemums (species of the gender Chrysanthemum - Asteraceae). The great advantage of the piretróides use consists of the low toxicidade of the same ones for mammals. That made to impel the use and the researches even our days.

The plants have been an important source of active chemical substances against insects, such as the nicotine, nornicotine and anabasine (alkaloids), the piretroids (piretrine and aletrine) and the rotenoids. (Crosby, 1971). Even so its direct use or of its gross extracts it is limited to domestic applications.

Insecticide natural products were very used until the decade of 40. Á to leave of that date, several synthetic biocidas appeared as by-product of the researches during Monday Great War, such like DDT, BHC, Aldrin, Dieldrin and Clordane, being those much more potent ones and also with smaller action especificty in the combat to the agricultural plagues that the natural ones, and they substituted like this completely the natural insecticides.

The return to the search of new natural insecticides, appeared due to the aleatory capacity of adaptation of the nature in relation to the indiscriminate use of synthetic

insecticides for the man and, the man's fact to have begun to reflect a little more on the relationship insect-plant. In that way, alerted by works as the one of Carson - I Liberate Silent Spring (1962) it started to respect a little more the natural mechanisms of adaptation.

For the use of insecticides botanical, several aspects they should be taken in consideration: extraction, conservation of the extracts, efficient dosagem, stability, toxicy and cost. All those aspects are understood when identified the main substances contained in this insecticide.

The main plants of the which are obtained substances with insecticide activities belong to the goods Nicotiana (*Solanaceae*), producing of nicotine and nornicotina; *Derris, Lonchocarpus, Tephrosia and Mundulea* (Leguminosae), producing of rotenoids, *Chrysanthemum* (Asteraceae), producing of piretrins and *Azadirachta* (Meliaceae), producing of azadiractine. The main forms of insecticide control made by plants can stand out the action fagoinibidora of the extract of the plant, which inhibits the feeding, but it doesn't kill the insect directly and the same ones are taken to the death by hunger. A substance can present action deterrent, which impedes the feeding of the insect.

The plants that present insecticide activities can stand out the class of the rotenoids (rotenone and tefrosine) that present activity, the piretroids (piretrines, jasmolines and cinerines) extracted of the flowers of chrysanthemums, the alkaloids (nicotine and nornicotine, metilcaconitine) that inhibit to the receivers of the acetilcolinesterase, the terpenóides (tenuline, angulatine) and mainly the azadiractina (isolated of the plant *Azadirachta indica*, known as neen) that possesses action interferente in the operation of the endocrine glands that they control the metamorphosis of the insects, impeding the development of the ecdise, presenting, still. Several they are the strategies capable to determine the activity of products of natural origin against insects and its isolation. In a general way, he/she begins with gross extracts of plants mixtures with several solvents (hexane, dichlorometane, ethyl acetate, methanol and water). soon after, the extracts assetses are fractional through the methods existent chromatography and the obtained fractions are retestadas, repeating the process until the obtaining do(s) composto(s) ativo(s). soon after it is chosen the bioensaio more adapted to determine the insecticide activity, that depends on the habits of the insects with which one wants to do the rehearsal. To determine the capacity of certain substances in repelling insects, mainly the treatment of the leaves of the plants is used, because it is known that the same ones are eaten by larvas of insects.

It is understood today that the combat to a certain plague cannot be made in an unilateral way. The control of insects should be integrated, with the feromônio use (associated to insecticides), natural enemies (through a biological control), natural products, deterrentes that induce mutations to reach the sterility in the next

generations of insects and other strategies that not look for the erradication of the insect-objective, but yes a balance to the already unbalanced ecosystem. The use of the quimiossistematic can aid in the obtaining of new natural insecticides, as the azadiractina, that can be considered as the most recent natural insecticide.

In that way, the great variety of present substances in the flora continues being an enormous attractiveness in the area of control of insects and plagues, mainly being taken in consideration that a small portion of those plants was just investigated with such an objective.