BEEHOLD

The colony of the honeybee (*Apis mellifera* L) as a bio-sampler for pollutants and plant pathogens

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Propositions

1. The focus of apicultural research on bee health veils the beauty of beekeeping and the multipotential application of the honeybee colony. (this thesis)

2. Unlike what is stated in the press and social media, the honeybee colony is an instrument to indicate and not the gauge of the quality of the environment.
(this thesis)

3. Solitary bees are excellent pollinators for crops, but their role will remain marginal in modern agriculture and horticulture pollination.

4. Developing adequate test protocols to evaluate the impact of chemicals on the environment offers more benefit to society than banning chemicals for political reasons.

5. The slogan "together we fight against cancer" incorrectly suggests one can beat cancer by fighting.

6. Experiencing sound as music or pothering noise is a matter of open minded ears and state of mind.

Propositions belonging to the thesis, entitled:

"BEEHOLD" The colony of the honeybee (*Apis mellifera* L) as a bio-sampler for pollutants and plant pathogens

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Thesis

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With this PhD thesis I honour Marlies.

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Chapter 1

General Introduction

Bio-indication with honeybee colonies (*Apis mellifera* L)

Paragraph 1.4 is a compilation of Steen, van der J.J.M. & Cornelissen, B. (2015). Factoren die het gedrag van honingbijen bepalen (deel I) Dracht in Nederland (Cultuurgewassen en wilde planten) (deel II). Rapport 606. Plant Research International Wageningen UR

Preface

"*Panta Rhei*" meaning "everything flows" or in other words "everything is constantly changing" is an aphorism of the work of the Greek philosopher Heraclitus (535 - 475 BC) made by Simplicius (560 - 460 BC). This constant change shows in the altering environment. Here is the genuine challenge of bio-indication; how to interpret the constant change and distinct between "normal" and "abnormal". The first prerequisite is to behold: observe carefully, record and interpret. Therefore, as this thesis is about the honeybee colony as passive sampling method (PSM), with a wink to the verb behold, the title is "BEEHOLD" The colony of the honeybee (*Apis mellifera* L) as a bio-sampler for pollutants and plant pathogens.

Bio-indication has many aspects ranging from recording of changes of ecosystems, of the inner state of organisms (bio-assay) to collection and accumulation of among others pollutants and plant pathogens by an organism. Applying honeybee colonies as a sampling tool is the latter mentioned form of bio-indication. Using a honeybee colony in environmental technology is where apidologie and environmental technology meet.

Apiculture is a world-wide industry. Honeybee colonies are managed all over the world except at the polar areas. This thesis may contribute to a further development and application of the honeybee colony for bio-indication. Especially in the regions where pollution is suspected or known, bio-indication by the honeybee colony can be a promising method because of its low costs and easy manageable way to detect pollution and plant pathogens.

I hope this thesis and the studies presented provide connecting factors for a further exploration of the honeybee colony as bio-indicator tool.

1.1 Introduction to the thesis

Bio-indication comprises a broad field with one common factor; a living organism is used to assess the incidence of, or hazard to, living organisms. In this thesis the possibilities and restrictions for bio-indication by the honeybee colony (*Apis mellifera* L) are presented and discussed.
Bio-indication is an environmental technology. Apidology and environmental technology are different specialities. Apidology, the knowledge of the honeybee and beekeeping, covers the broad range of managing honeybee colonies, the biology of the insect *Apis mellifera*, honeybee diseases and the interaction between environment and vitality of the honeybee and the honeybee colony. The definition of environmental technology according to the European Union as stated in the Environmental Technology Action Plan (ETAP) is a technology to improve the environment (end of pipe technology) or an alternative technology that has less impact on the environment (EC-Europe). Environmental technology comprises indication and monitoring of pollutants in the environment, the study field. In apidology the environment is where honeybees collect their food. This is where both specialities meet as it is the same environment. In environmental technology terms, the colony acts as a bio-sampler of pollutants, indicating pollutants in the environment. The features of the honeybee and beekeeping practices serve environmental technology.
A foraging honeybee collects nectar and pollen from flowers, honeydew on leaves and needles of coniferous trees, water on flowers, leaves, plants and ponds and propolis on buds. During the active season in the field, which ranges in the Netherlands from March / April until September / October and year-round in greenhouses, hundreds to thousands of bees depart from a honeybee colony daily for collection flights. On each collection flight dozens of flowers are visited by the individual foraging bee. Along with the collection of food and propolis, particles atmospherically deposited on the foraging sites or present in the flowers are collected unintentionally. Each forager acts as a micro-sampler, accumulating her micro-samples in the colony. The phenomenon of collecting and unintentional and passive accumulation target matter makes the honeybee a bio-indication tool. Applying the honeybee colony for bio-indication can be considered as a Passive Sampling Method (PSM). Bio-indication, its definitions, application and historical context are presented in paragraph 1.2.
The Source-Path-Receptor concept (SPR) is applied in environment technology. SPR is used to identify where in the source-path-receptor process the honeybees might encounter and collect target matter for bio-indication (paragraph 1.3). To understand the tool "honeybee colony" for

bio-indication study, the features of the honeybee colony in its foraging strategy, amounts food collected, collecting frequency, dispersal of the bees over the foraging site per colony and per apiary are described in paragraph 1.4. The state of the art of the bio-indication by the honeybee colony is presented in paragraph 1.5. To obtain collected target matter from the honeybee colony for analysis, it must be subsampled. Subsampling of a honeybee colony can be done sacrificially and non-sacrificially. Sacrificial subsampling means bees are sacrificed for analysis and in applying non-sacrificial subsampling no bees are taken from the colony and target matter is obtained from the bee's exterior. Definitions and applications of both sacrificial and non-sacrificial subsampling of the honeybee colony are given in paragraph 1.6. For reliable results of a bio-indication study, the factors target matter, target matter location (source-path-receptor), location of the study site, sampling methods and sample sizes must be taken into account. The flow chart with the seven critical steps for bio-indication with the honeybee colony is presented in paragraph 1.7.

Three studies in which the honeybee colonies have been sampled sacrificially and three studies in which the honeybee colonies were sampled non-sacrificially are presented. In the three bio-indication studies on heavy metals: a study on the spatial and temporal variation of heavy metals in honeybees, a study about the relationship between heavy metals in ambient air and in honeybees and the national surveillance study on heavy metals in honeybees, the colonies were subsampled sacrificially (Chapter 2, 3 and 4). For non-sacrificial subsampling of the honeybee colony a new device has been developed. The Beehold device in which the Beehold tube is the actual sampling part, samples non-sacrificially hive-entering bees by forcing them to enter the hive via a tube, internally lined with a moderate sticky material to which particles on the bee's exterior adhere to the Beehold tube. The Beehold device and its application are described in Chapter 5 in the *Erwinia pyrifoliae* study in a flowering strawberry greenhouse cultivation. Also in the studies on detection of *Erwinia amylovora* in flowering fruit orchards in Austria and the bio-indication study on γ-HCH in the Bitterfeld region in the eastern part of Germany, non-sacrificial subsampling was applied (Chapter 6 and 7).

In the general discussion, the pros and cons of the PSM honeybee colony, based on the biology and features of the honeybee and the honeybee colony and the applicability to detect heavy metals, plant pathogens, γ-HCH and investigation of the foraging area, are discussed in Chapter 8. Safe subsample sizes, meaning sampling of honeybees without affecting significantly the colony's development and performance depend on the

colony size. Sample sizes and number of colonies for a representative local study result are discussed. Non-sacrificial subsampling has no subsample size restriction but does have an under limit, under which the chance of detecting target matter is low. The 7-steps frame work is discussed step by step followed by a proposed practice taking into account the possibilities and the restrictions of the PSM honeybee colony.
Bio-indication with the honeybee colony is an underexplored study field. The general discussion ends with suggestions for further research.

1.2 Bio-indication, definitions & brief overview

1.2.1 Definitions

Bio-indication is the application of organisms for the detection of alterations of the environment. Bio-indication implies both collection and accumulation of matter regardless of the impact on the organism and recording of changes of the organisms as a result of exposure to (toxic or pathogenic) matter.
Stöcker (1980) defined bio-indication as a time dependant, sensitive registration of anthropogenic factors or anthropogenic altered environmental factors by distinguishing dimensions of biological objects and biological systems under definable circumstances. In itself bio-indication is the result of two functions: environment and history of the organism. The definitions of bio-indicators and bio-monitors according to Markert et al., (2003) are: a bio-indicator is an organism (or part of an organism or a community of organisms) that contains information on the quality of the environment (or part of the environment). A bio-monitor is an organism (or part of an organism or a community of organisms) that contains information on the quantitative aspects of quality of the environment (or part of the environment).
In traditional biology (Natural History) indicator organisms are applied to measure effects of environmental changes such as alteration of the habitat, habitat fragmentation both temporal and spacial. In ecology, bio-indication is used in a wide range of toxicology studies ranging from LD_{50} tests, single species microcosm, and mesocosm studies, to practical field trials as a tool to record the impact of e.g. new chemicals on organisms and populations.

1.2.2 Bio-indication

During evolution organisms, populations, biocenoses and complete ecosystems are influenced and adapted to numerous biotic and abiotic stress factors like climate fluctuations, radiation, food supply, predator-

prey relations, parasites, diseases and competition between and within species. Due to human activity many xenobiotic substances have entered the environment in a relatively short period, in particular after the industrial revolution that started in the 18th Century. This influx of xenobiotic compounds affected the environment and posed a stress factor to organisms. The effect of anthropogenic influences can be monitored with satellites, instrumental techniques plus by observing and recording changes on and in organisms, from individual organisms to ecosystems: bio-indication. Recording varies from changes of populations and phenotype to exceeded loads of anthropogenic materials in and on organisms. The first description of the link between atmospheric pollution and damage to trees dates from about 2000 years ago. Pliny the Elder (23-79 AD) described in his "Historiae Naturalis" the damage to the needles of coniferous trees at locations where iron oxide was made from iron sulphide. Later, at the end of the 17th and beginning 18th Century, fish mortality in the rivers Rhine and Thames were attributed to water pollution. In the 20th Century the concept of a malleable and controllable environment came up with the highlight of the landing of Apollo II on the moon, the ultimate victory of man and man's technique over nature. Rachel Carsons's book "Silent Spring" caused a turnaround of this concept. She showed that survival of mankind depends on nature and unlimited destruction and altering of the environment would affect mankind (Markert et al., 2003). In 1986 the accident at the Chernobyl Atomic Energy plant in the Ukraine showed how supposedly controllable processes, can by accident turn into uncontrollable processes. The effects of the radioactive fallout after the Chernobyl accident affected large parts of Europe. For example, in the North of Scandinavia radio-active Cs137 could be detected in among others lichen, the main winter staple of the Scandinavian reindeer, making the reindeer meat unmarketable (Blackwell, 2003). In the Netherlands Cs137, in amounts of 230 – 1000 $Bq.kg^{-1}$ was detected in *Paxillus involutus* (gewone krulzwam) (Oolbekking & Kuyper, 1989). More recently in 2011, an earthquake followed by a tsunami damaged the Fukushima Dai-ichi nuclear power plant in Japan. This event significantly increased Cs137 levels in the groundwater, coastal sediments and ocean near the discharge point (Buesseler et al., 2011).
The indicative feature of a biotic system, from individuals to ecosystems is determined by inherent physiological characteristics, population dynamics and environmental stress by physical and chemical alteration of the environment. The response is often not specific. Therefore, bio-indication results mostly in a general warning which may indicate causal links. Bio-indication operates by definition during the entire exposure period. The disadvantage is a variable response of bio-indicator organisms. Bio-

indicators can be grouped by test organisms, indicator organisms and monitor organisms or by effect indicators and accumulation indicators. For atmospheric pollution detection, bioindicating plants are used most commonly. Mosses accumulate heavy metals and xenobiotic substances. As mentioned before, lichen accumulates radioactive compounds in large quantities; lichen has no excretion organs. In plants SO_2 affects stomata regulation resulting in the disturbance of the metabolic processes. Coniferous plants like *Abies alba*, *Pinus sylvestris*, *Picea excels* reacts on the SO_2 exposure (Fränzle, 2003). SO_2 is part of particulate matter $PM_{10/2.5}$. Particulate matter is composed for approximately 25% of inorganic compounds (SO_2 sulphate; NO_x nitrate oxides; NH_3 ammonium), 12.5% of carbon compounds, both elementary carbon and organic carbon compounds, 12.5% of sea salt aerosol (NaCl), 12.5% of oxides of Si, Al, Ca, Fe and K mostly from soil erosion caused by human activity and resuspension of road dust and 37.5% water. Most of the inorganic compounds have an anthropogenic origin (Buijsman et al., 2005). In contrast to plants, animals have mechanisms to cope with environmental stress. The ability to translocate themselves is a feature plants don't have. In general, primary consumers are better bio-indicators compared to secondary consumers because the primary consumers live on a relatively low energy level and have to consume large quantities. Secondary consumers consume food with a higher energy level and consume less quantity (Fränzle, 2003).

1.2.3 Bio-indication and politics

In France at the end of the 19th Century, pesticides were developed to protect the viticulture followed by development and mass production of pesticides in the 20th Century. The primary focus was on the pests and not on the side-effects. The public awareness and interest for the environment came up in the 50's and 60's of the 20th Century. Progressing scientific knowledge about control of emission of xenobiotic substances and monitoring programs to signal side-effects started then. Since then, in the industrial world, "environment" is a political factor. To protect man, animal, plant and landscape, public tax money is spent. Politicians require information about the quality of the environment to take precautionary or remedial measures and to evaluate the result of political decisions. This applies both to chemical pollution and alteration of the environment. At the United Nations Convention on Biological Diversity (Rio de Janeiro 1992) environmental protection and socio-economic development were the key issues. At the Gotenburg EU Summit (2001) it was agreed to aim for prosperity for present and future generations and a holistic approach

of links and synergism between economics and environmental dimensions of politics. At the European Union Treaties of Maastricht and Amsterdam the precautionary principle was accepted. This principle says that in case there are indications a substance poses a risk; it can be forbidden without scientific evidence. Public health protection is nowadays set by acceptable and non-acceptable limit values of xenobiotic substances in the atmosphere, water, soil and food. Bio-indication is a tool within this process (Kienzl, et al., 2003). The OECD (Organisation of Economic and Cultural Development) sets standard protocols for bio-indication. Generally, results of bio-indication studies should give a clear picture of the condition of the environment, it should be easy to interpret, is must show trends and alteration of the environment by mankind, it should provide a basis for international comparison and set reference values to indicate significant deviations (OECD).

1.2.4 Bio-indication and honeybees

Honeybees are included in the broad field of bio-indication, ranging from ecotoxicology study in the field of ecology to indicating qualitatively environmental pollution and plant pathogens. Ecotoxicology comprises a range of honeybee tests to assess the impact of chemicals / pesticides, both currently legislated and applied and new ones in the legislation process. Without being complete I mention the range of tests applicable. Honeybees are used as a reference for pollinating insects. Since the 50's of the 20th Century, first and higher tier study protocols have been developed to assess the impact of pesticides both on the individual bee and on the honeybee colony. LD_{50} tests (first tier) are performed to determine the toxicity of a substance. Higher tier tests such as tunnel and field trials are performed to assess the hazard of chemicals to the honeybee colony. The hazard depends on exposure route, duration and the concentration of the chemical tested (Oomen & Thompson, 2010). In the same context, physiology is also a bio-indication parameter. Among others, assessing the concentration and course of the seasonally variation of vitellogenin, an important storage glycoprotein in honeybees is part of it (Steen et al., 2015). Applying honeybee colonies as indicators of environmental pollution and plant pathogens, the subject of this thesis, is part of the broad bio-indication spectrum.

1.3 Environmental pollution and plant diseases: Source–Path-Receptor concept

In order to define adequate bio- and chemical remediation measures, in environmental science the Source – Path – Receptor (SPR) concept has been developed. The SPR concept describes the source of an environmental pollution, its path through the environment and its receptor. The bioavailability of pollutants determines whether there is a risk for specific receptors and to what extent remediation can or should be used (Grotenhuis & Rijnaarts, 2011).
In this thesis subjects of bio-indication are denoted as target matter. For bio-indication of persistent organic pollutants (POPs), heavy metals and plant pathogens, the SPR concept is applicable. The target matter can be bio-sampled at its source, its path through the environment and at its receptor. The SPR of POPs is described on the basis of hexachloro-cyclohexane (HCH). For the SPR of heavy metals, these metals are described as a group with some examples. The SPR of plant pathogen is described on the basis of the bacterium *Erwinia amylovora*. The SPR for the three target matters is described in the order: structure, toxicity / pathogenesis, source, path and receptor.

1.3.1 Hexachlorocyclohexane (HCH)

HCH ($C_6H_6Cl_6$) is a hydrophobic chlorinated molecule (molar mass 290.8 $g.mol^{-1}$; water solubility of 2.5 $x10^{-2}$ $mg.L^{-1}$) (Briand et al., 2002). HCH is produced as technical HCH (65-70% α-HCH, 7-20% β-HCH, 14-15% γ-HCH, 6-10% δ-HCH, 1-2% Σ-HCH) and as the pesticide Lindane (99% γ-HCH) (Popp et al., 2000). γ-Hexachlorocyclohexane, affects the neuro system, liver and kidneys and bio-accumulates in the fat tissue of organisms. Relevant for bio-remediation, organic contaminants may be toxic to micro-organisms. The toxicity may be related to the octanol-water participation coefficient (K_{ow}); organic solvents having a log $K_{ow} > 4$ are not toxic as a log $K_{ow} < 2$ are toxic (Grotenhuis & Rijnaarts, 2011).
The sources of HCH in the environment are contaminated soil and groundwater. In the soil of contaminated sites, POPs are bound to soil particles and are present in pore- and groundwater. The availability depends on non-equilibrium conditions between pollutants bound to mineral soil particles, pore water, vapour in unsaturated zones and groundwater. Limited availability is believed to be the result of long aging procedure in which soil organic matter and soil area of small sized particles like clay play a role. Between source and receptor is a plume of contaminants varying from meters to kilometres. Fluxes from soil to surrounding water and next for uptake in the food chain pose a risk

(Grotenhuis & Rijnaarts, 2011). The potential bioavailability fraction of HCH is high (>70%) even after extensive aging. The high availability combined with the low degradation forms an environmental risk (Smit et al., 2005).
HCH was produced until in 2009 the production was forbidden except for medical purposes (lice and scabies treatment). Between 1950 and 2000 worldwide approximately 600,000 tonnes Lindane was produced (Wikipedia / Lindane). In the Bitterfeld region in Germany (e.g. Chemie Kombinat Bitterfeld) Lindane was produced from 1951 – 1982. The undesired by-products (α, β, δ-HCH) were dumped in landfills. HCH is also a waste by-product of various manufacturing processes like cable manufacturing and smelting of PVC sheeted cables (Popp et al., 2000; Manz et al., 2001). Lindane was used in agriculture, in forestry, veterinary medicine and for disinfection of storage rooms. Due to its persistence HCH is still found in soils (Manz et al., 2001).
The path of HCH contamination is partly via air and mainly via soil. The atmospheric deposition is via volatility and wind erosion of contaminated soils (Briand et al., 2002). In 1998 Popp et al. (2000) measured HCH in the atmosphere (gas + particle bound portions) in Leipzig, Roitsch and Greppin in the former GDR. Detectable amounts in ng.Nm^3 were found.
β-HCH was the main component measured. The highest concentration measured in Greppin is due to the former chemical plants and landfill dumps in the Bitterfeld region. β-HCH, one of the isomers formed during the Lindane production shows, despite its low solubility, a high mobility in polluted soils. There is a positive correlation between dissolved organic matter (DOM), mobilisation and transport of β-HCH. β-HCH can be detected in deeper soil horizons because of the coupling of β-HCH / DOM (Kalbitz et al., 1997). Contaminated groundwater interacts with local streams; POPs are released from adjoining aquifers into the stream and streambed sediments. These streambed sediments are the dominant contamination source for surface water. Turbulent conditions like flood events result in an increase of desorption of POPs from sediment to surrounding water. Hydraulic retention time (HRT) and particle size determine the concentration gradient (Smit et al., 2008).
Due to the mobility of the aged HCH pollution from groundwater to surface water, streambeds and flood plains sediments are receptors of HCH (Heidrich et al., 2004). Soil erosion followed by atmospheric deposition of HCH containing soil particles may result in contamination of among other things, vegetables and flowers.

In figure I the source-path and receptor of HCH is schematically shown.

Figure I. Source: contaminated soil. Path: from soil to surface water, next to sediment and next resuspendation via wind erosion into the air. Receptor: surface water, atmospheric deposition of POPs containing particles in e.g. vegetables, houses, flowers and playgrounds.

1.3.2 Heavy metals

There are multiple definitions of heavy metals: all metals having a higher atomic mass than iron; all elements between copper and bismuth in the periodic table; all toxic metals. Biota require some heavy metals in trace quantities for vital processes, however, in large quantities heavy metals are toxic. The biological availability depends on the speciation. The toxicity of heavy metals is largely due to their reaction with the sulfhydryl group of enzymes. This reaction inhibits enzymes by masking catalytically active groups by protein denaturation or by altering substrate sites. Lipophilic organic metallic compounds pass the blood-brain barrier, causing neurotoxicity (Niesink et al., 1996; Karaca et al., 2010). Exposure of 0 – 4-year-old children to lead (Pb) results in an increased level of Pb in the blood. Concentrations of 20 – 40 µg.l blood^{-1} decreases the IQ by 1 point (Wezel et al., 2008).

Heavy metals occur naturally in soils as trace elements. Increased concentrations of heavy metals in the environment are the result of human activity. Fossil fuelled transport, combustion, mining, various metallurgical processes, agricultural activities and leaching / oxidation of metal structures are the main sources. Road transport is the main source of airborne mineral dust and heavy metals deposition in urban areas due to non-exhaust emission: road dust resuspension and brake- and tyre wear. Vehicle related components are Fe, Bi, Sn, Sb, Ba, Cr, Cu and Zn. Brake tracers are Cu, Fe and Al. Zinc is a tyre tracer (Amato et al., 2013). In the soil, heavy metals are bound by soil particles. However, due to changing land use heavy metals may leach into the soil water phase and become available. In situ chemical oxidation (ISCO) applied for

remediation of contaminated soils of POPs can have the unwanted side-effect of mobilisation of heavy metals as oxidation of organic matter decreases the binding capacity (Grotenhuis & Rijnaarts, 2011). Quinton & Catt (2007) demonstrated that water erosion on agricultural soils, having only received agrochemicals, resulted in enriched metal concentrations in the sediment up to toxic levels. Mean concentrations of Cr, Cu, Pb and Ni were up to about four times higher in sediment than in the parent soils. All sediment heavy metals concentrations were significant correlated with clay and silt sized fractions of the sediment and the carbon content. Clay particles measure < 2 µm, silt 2 – 50 µm (Bouyoucos, 1962). Robert & Johnson (1978) demonstrated that wind erosion of metal contaminated soils in the vicinity of a mining complex resulted in dispersal of metal waste material. Soil Pb and Zn decreased exponentially with the distance downwind of the spoil heaps. The abundance of Pb and Zn close to the spoil heaps reflected largely the chemical composition of the waste material. A study of elemental composition of street dust in Spain revealed a pattern of Zn, Cd, Hg and Pb in street dust, almost identical to urban soils, demonstrating wind erosion dispersal of soil particles. The proximity of industry (zinc melting activity) and Zn, Cd and Hg concentration in street dust were positively correlated (Ordonez et al., 2003). A similar pattern was demonstrated by Charlesworth et al., (2003) in Birmingham and Coventry; brass and coin making activities were identified by higher concentrations Cd, Zn and Cu in the proximity of these industries. Concentrations of Zn and Cu in street dust showed a positive correlation with traffic. The size distribution of aerosols from soils show strong resemblance to the size distribution of the soil itself; for particle size of $0.1 \leq r \leq 1$ µm, the fraction of soil particles increases with decreasing radius and particles of $1 \leq r \leq 6$ µm show a decreasing slope of -2. Particles < 1 µm are more present in aerosols than particles > 1 µm. Clay particles tend to agglomerate to other particles forming bigger particles (Gillette et al., 1972).
In figure II the source-path and receptor of heavy metals containing particles is schematically shown.

Figure II. Source: Industry and traffic. Path: air, deposition on soil and next resuspendation via wind erosion into the air. Receptor: atmospheric deposition of metal containing particles in e.g. vegetables, houses, flowers and playgrounds.

1.3.3 Plant pathogens

The SPR of plant pathogens is diverse. The SPR of *Erwinia amylovora*, the bacterium causing fireblight, is described as an example of SPR of plant pathogens. *E. amylovora* is a Gram negative, facultative anaerobe bacterium of the *Enterobacteriaceae* family. The straight bacterium rods measure 0.5 to 1 x 1.0 to 3.0 µm and are mobile with flagella. *E. amylovora* causes fireblight, a necrosis in many species of the *Rosaceae* plant family. The annual life cycle of *E. amylovora* is associated with living host plants. In spring the bacteria start to multiply and form primary inoculums at the edges of overwintering cankers formed the previous year. This primary inoculum is disseminated by wind, insects, bird or rain and enters host plants via natural orifices of flowers, via lenticels, via stomata and wounds mainly caused by hailstorms and/or insect feeding and oviposition. In the blossom, bacteria multiply in the nectar. From the flower, the bacteria move into the branch. In the host plant the bacterium multiplies intracellularly, killing the cells that turn dark. Under moist, warm circumstances an exudate of polysaccharides and *E. amylovora* bacteria is formed which poses a blossom-, shoots- and fruits infection risk. Under dry circumstances so called "strands" are formed. These needle-like strands are disseminated by wind. At the end of the growing season typical overwintering cankers are formed in which the bacterium hibernates (Zwet & Keil, 1979 and Deckers, 1982 in Wael, 1988; Colorado State University Extension, 2014). In figure III the Source-Path-Receptor route of *Erwinia amylovora* is schematically presented.

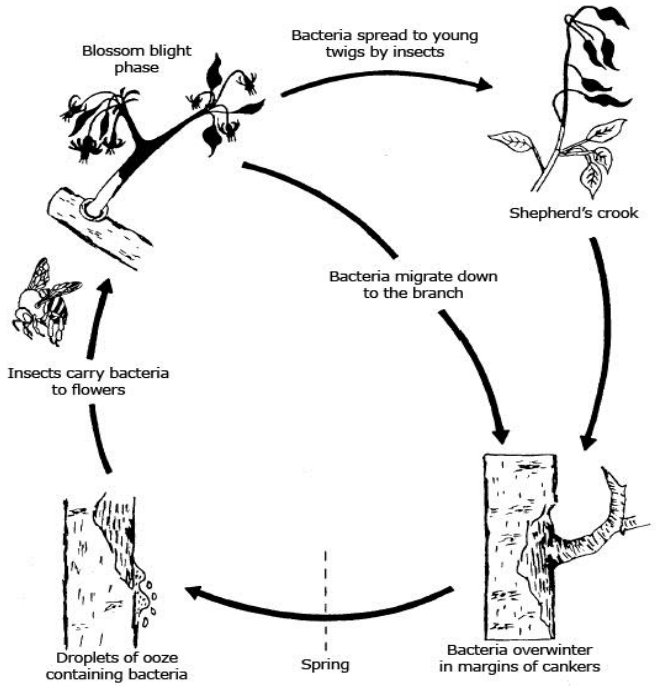

Figure III, SPR of *Erwinia amylovora* (http://www.ext.colostate.edu/pubs/garden/02907.html)

1.3.4 Source-Path-Receptor approach applied to the bio-indicating honeybee colony

Based on the SPR concept, for bio-indication of the γ-HCH by honeybee colonies, atmospheric deposition of γ-HCH containing sediment particles on trees and flowers is the main location. For heavy metals both, interception of re-suspended metal containing particles of road dust and metal containing combustion particles metal containing soil particles in the air by wind erosion and next atmospheric deposition of metal containing particles on trees and flowers are the locations bees can collect these particles. Plant pathogens, like *Erwinia amylovora* and *Erwinia pyrifoliae,* are collected both from flowers containing pollen and nectar. The source, path and receptor can be in a single flower or multiple flowers. In the bio-indication of airborne plant pathogens, flowers and leaves with honeydew are the receptor where honeybee may collect the micro-organisms.

1.4 Features of the honeybee and honeybee colony in perspective of bio-sampling of pollutant and plant pathogens

1.4.1 The honeybee colony

The honeybee colony is considered a superorganism, showing numerous analogies to multicellular organisms. Sterile workers fulfil the role of somatic cells in organisms with intricate and complex interactions. These interactions are under partial control of hierarchical signals used for global information of the colony. The majority of the activities in the colony are regulated through local decision making and through self-organising processes, regulated by workers threshold response variability. The colony level selection is predominant over the individual selection force, similar to organismic selection where selection among cells or within cells is less relevant to evolutionary processes than fitness at the organismic level (Moritz & Fuchs, 1998).

The honeybee colony consists of one reproductive bee, the queen, thousands of female bees (workers), and in summer hundreds of male bees (drones). The worker caste includes four sub-castes: cleaning caste, brood nest caste, food storage cast and foraging caste (Seeley, 1983). A honeybee worker lives for four to five weeks in the summer and for six to eight months in the winter. In the moderate climatic zone of the northern hemisphere, the colony is actively foraging from April to October, although this period shows variation depending on weather conditions and local circumstances. Facts and figures presented are about the active foraging period. The tasks of the workers are age-related. Globally, during the first three weeks the workers are in-hive bees, cleaning the cells (cleaning caste), nursing the brood, queen, drones and young adult workers (brood nest caste), defending the colony, transferring the incoming food to the cells and other bees and processing the nectar into honey (food storage caste). In the last one to two weeks of her life the worker bee is a forager, collecting food (foraging caste). During the active foraging and breeding period of the colony, approximately 25 to 40% of the population is potentially a forager bee. The honeybee colony is a symbol of an efficient, cooperative community in which every bee is busy, contributing her share to the welfare of the colony. In reality, however, this is not entirely correct; about 10 – 30% of workers inside the hive shows no specific activity, they are the resting bees (Seeley, 1995). This pool of resting bees has its function. They are spare bees. The age-related tasks are not cast in concrete, but show plasticity. If the composition of the worker pool or the tasks to be performed changes dramatically, the worker bee's activities can be adjusted. For instance, older bees can regenerate their

food glands to become nurse bees again if there is a sudden shortage of nurse bees, and young in-hive bees can start foraging activities before they would normally forage if a significant number of foragers were to disappear (Winston & Fergusson, 1985; Free, 1967). The tasks are more related to the need of the colony than to an individual bee's age (Winston & Punnet, 1982; Moritz & Fuchs, 1998).

1.4.2 Foragers

Honeybee colonies vary in size of about 7000 individuals in spring to 20,000 – 35,000 bees in summer, decreasing in size in autumn towards the hibernation. In summer, there can be up to 35,000 worker bees in the colony. As a consequence, the number of foragers depends on the colony size. The total number of bees in a colony can be assessed quickly as on one side of a honeycomb when the bees are side by side there will be 125 bees per dm^2 (Delaplane et al., 2013). In Table 1, estimations of the total number of bees on fully occupied 1, 5, 10 (one storeys hive), 15, and 20 (two storeys hive) Simplex measured frames is presented.
The measurements are done at the normal brood nest temperature of 34 – 35° C.

Table 1. number of bees in a Simplex hive (inner frame measures 340 x 198

Frames 2-sided fully occupied	dm^2	Number of bees
1 frame	13.5	1683
5 frames	67.3	8415
10 frames	134.6	16830
15 frames	202.0	25245
20 frames	269.3	33660

The percentage of foragers (four to five week old bees) in a colony is maximally 40%. In a strong colony this is approximately 10,000 foragers, but not all foragers are constantly foraging. The lifespan of a forager is, on average, seven to eight days with a minimum of about five and a maximum of nineteen days (Visscher & Dukas, 1997; EFSA, 2014). In this period the bee can fly approximately 800 km (Neukirch, 1982). The bees forage for nectar up to 13 km, for pollen up to 6 km and for water up to 3 km (Steffan-Dewenter, 2003; Visscher et al., 1996). The flight speed ranges from 4.9 to 8.2 meter per second (Osborne et al., 1996; Riley et al., 2005; Gmeinbauer & Crailsheim, 1993). The flight altitude is one to two metres (Esch et al., 2001). A forager makes about ten foraging flights per day, being on average away for ten minutes for nectar and thirty to eighty minutes for pollen (Winston, 1987). The collection process is the result of three sub-processes 1) scout bees find new sources and recruit foragers to exploit these sources; 2) scout bees only recruit foragers for

profitable sources; 3) foragers stop collecting nectar and pollen on sources that are no longer profitable (Visscher & Seeley, 1982; Frisch, 1967, Heinrich 1978 in Seeley, 1985). The distance bees forage for nectar and pollen depends on the availability of the food sources and on the energy it costs to collect the food. The energy costs for foraging are about 6.5 $J.km^{-1}$. In order to collect the annual need of 125 kg nectar for energy and 20 to 30 kg for their protein, minerals and fatty acids requirements, approximately 4,000,000 nectar foraging flights and 1,125,000 pollen foraging trips are made. The amount collected during a trip depends on the distance between the hive and the nectar source and on the nectar sugar concentration. The closer the nectar source the less nectar is collected per trip and the same is true for low sugar containing nectars. Bees go for the most efficient way and calculate the benefit, taking into account the flight costs and the profit (Seeley, 1985). When foraging time and energy profitability are equal, the energy profitability determines where to forage (Schmid-Hempel et al., 1985). On foraging sites of equal nectar value, the nighest site is preferred (Ribbands, 1949).

1.4.3 Nectar, pollen and water

Honeybees mostly depend on flowers for their food. The food components are collected during foraging flights. Bees collect nectar (the sweet secretions of the nectaries), pollen, honeydew (the sweet secretion of aphids) and water for their own direct needs and store the surplus for winter survival. Propolis is collected in little amounts for hygienic purposes. Food collection is organised via scout bees that look for food, bringing back the message and recruit bees for foraging. Scouts bringing in the best quality and quantity food, both nectar and pollen will recruit more foragers than scouts bringing in less quality and quantity food. The foraging behaviour is constantly adapted to the needs of the colony and the attractiveness of the nectar- and pollen source. In the foraging process, the frequency of the trips is determined by the time it takes for foragers to unload their harvest to the bees inside the hive. A forager bringing in nectar with a relatively high concentration of sugar will more quickly find a bee of the food storage cast that is willing to accept her nectar load than a bee bringing in less attractive food. In this way colonies focus on high yielding foraging sites (Farina, 1996; Tezze & Farina, 1999). The in-hive food collection caste consists of 18 – 28 day old bees (Brodschneider et al., 2007; Seeley, 1995). As honeybees have only small energy reserves in the body, they depend on the amount of sugar in the honey sac (proventriculus). This sugar is transferred to the ventriculus and across the ventricular cell wall into the haemolymph down a concentration gradient. Ergo, the sugar concentration in the honey sac determines how far a bee can fly (Crailsheim, 1988[a]; Crailsheim, 1988[b]).

During a foraging trip, up to hundreds of flowers can be visited collecting 25 – 40 mg nectar (21 – 33 µl) per trip by nectar foragers and 10 – 30 mg pollen by the pollen foragers. Each pollen forager carries two pollen pellets. On average a pollen pellet weighs 6 – 11 mg (Maurizio, 1953). As nectar- and pollen flow depend on plant- and climate conditions, the number of flowers visited and number of foraging trips can only be estimated. The regulation of pollen foraging is based on a feed-back system via trophallaxis. High protein content in the jelly, fed to the pollen collectors by the nurse bees, incites the bees to decrease foraging for pollen and to switch to nectar (Camazine, 1993; Fewell & Winston, 1992; Free, 1967). In spring more pollen is collected than in autumn. The amount of pollen collected, is positively related to the amount of brood. In the same way as for nectar, bees focus on profitable pollen sources. Most pollen has an incomplete essential amino acid pallet. Honeybees need pollen diversity to fulfil their need for a complete protein diet (Groot, 1953; Alaux et al., 2010; Di Pasquale et al., 2013). The nurse bees, the age class of about 4 – 14 days old in-hive bees, consume the pollen and produce jelly to feed the larvae, queen and young bees. Annually, a honeybee colony needs 25 kg water for dilution of the larval food and for cooling the brood nest (Nicolson, 2009; Kühnholz & Seeley, 1997). As water has no energy input for the return flight, bees return from these trips on their energy reserves, which restricts the flight distance to 2 to 3 km (Visscher et al., 1996). During a foraging trip bees show flower constancy and location constancy. Flower constancy means that during a trip, the bee restricts herself to one (plant) species and keeps on doing this until the resources are 'dry' or a better alternative shows up. A bee can remember a good source for months (Menzel et al., 2005). The flower constancy is not absolute; up to 11% of pollen foragers collect pollen of different sources (Free, 1963; Maurizio, 1953). Also the division between pollen and nectar collectors is not absolute. In a study conducted by Ribbands (1949) 58% of the bees collected only nectar, 25% collected only pollen and 17% collected both.

1.4.4 Communication: a means to efficient foraging

The highly efficient collecting of food is achieved by effective communication systems which are geared towards the benefit of the colony rather than towards the individual bee. The communication systems are the well-known bee dances and trophallaxis (food exchange). By dancing, the location of, the direction of and the distance to nectar, pollen and water sources are communicated to the food collectors by the scout bees. Of all available forager bees, 13 to 23% are scout bees. This percentage will increase in case there is food shortage and decrease in case food is available abundantly (Seeley, 1983, 1985). Not all recruited

foragers will find the source that has been communicated. Approximately one third will find the source immediately (Mautz, 1971). It takes a bee around 2.4 and 4.8 trips to locate a source at 200 metres and 1000 metres respectively (Seeley, 1983).

1.4.5 Exploring and exploiting the foraging area

Theoretically the maximum foraging area for nectar is 450 km^2, for pollen is 113 km^2 and for water is 28 km^2. Bees prefer to collect their food as close by to the hive as possible (< 1 km). Because of the link between distance and profitability, every meter further away from the hive than needed costs energy and is only worth flying in case the profit is higher than the energy cost. The maximum distance will only be flown for very profitable sources or if no other sources are available in the proximity. On rich sources a single bee exploits 10 – 40 m^2, on less profitable sources the distance between visits increases and the bees become more restless (Ribbands, 1949; Butler et al., 1942, Sing, 1950, Weaver, 1957 in Seeley, 1985). A food source is effectively exploited by the individual bee by considering direction and distance. The less sugar in the nectar and the less time the bee spends on a flower, the less change in direction from one flower to another is observed. Ergo the richer the source and longer the time spent on one flower, the more bees change direction flying from one flower to another. Decreasing directionality to less profitable sources helps the bee to spend less time on these sources (Schmid-Hempel, 1984; Waddington, 1980). The foraging behaviour of honeybees shows seasonality. In spring, bees will dance to indicate nectar sources of about 30% sugar, in summer it takes higher sugar concentrations and in autumn lower. Also in spring the foraging distances are shorter than in summer and autumn. In spring, bees have a thorax temperature of 35.7° C and in summer 26.8° C. The higher temperature in spring protects the bee against cooling down at lower ambient temperatures. The higher thorax temperature in spring costs more energy at the expense of the distance (Kovac & Schmaranzer, 1996).

1.4.6 Dispersion of honeybees of one colony over the foraging area

It is obvious that, given the focus on profitable nectar and pollen sources and the effective communication systems, foragers of one colony do not forage homogeneously over the foraging area. In fact, only part of the theoretically available foraging area is exploited. This area can change daily or even over a few hours (Visscher & Seeley, 1982, Seeley, 1985; Ribbands, 1949). In an orchard, a single forager will restrict herself to one or two flowering trees in the same row (Free, 1966; Free, 1974). This process results in a partial coverage of the foraging area. Colonies in one apiary can forage on similar, different and overlapping sites. It is common

knowledge among beekeepers that even in case a colony is located next to a profitable crop, there will always be bees coming in with pollen from other crops. This is the result of the competition between scout bees. Each scout bee can recruit a limited number of recruits. The bees that are in the proximity of the scout bee inside the hive will receive the dancing- and trophallaxis message of the scout bee, the others not. Additionally, recruited bees that are unable to find the location turn into scout bees looking for new food sources in the proximity of the location they were directed to (Mautz, 1971). As a result of recruiting and focus on highly yielding nectar- and pollen plants, a relatively small number of nectar- and pollen producing plants, will be visited. During a 7 days period an average of 10 different crops are visited daily (Visscher & Seeley, 1982; Frisch, 1967, Heinrich 1978 in Seeley 1985). Garbuzov et al. (2014) decoded the waggle dance of three colonies in an urban area (Brighton). It appeared that about 90% of the foragers visited food sources within 1 km although further away also good food sources were available.

1.4.7 Dispersion of the bees from colonies in an apiary over the landscape

Colonies placed in the same apiary will visit partly the same crops and partly different crops in the foraging area. Waddington et al. (1994) studied the dispersal of foragers of two adjacent colonies based on the bee dances. It appeared that the colonies visited mostly different sites. This changed daily. A plausible explanation is that one colony finds the crop first, exploits it and the next colony will find a crop that is already exploited. This colony search for unexploited crops and the different needs per colony for nectar and pollen will probably contribute to this phenomenon. The gradient in which colonies disperse themselves over the landscape is rather unpredictable as the mechanisms are not yet fully understood.

1.4.8 Consumption of honey and pollen

In temperate climates, honey is produced from nectar and honeydew (secretion of aphids). Both nectar and honeydew originate from the phloem sap of higher plans. In addition to carbohydrates, nectar and honeydew also contain organic acids, vitamins and minerals. The natural mineral content of phloem sap consists mainly of potassium (K), as well as minerals like sodium (Na) and magnesium (Mg) which are detectable in very small amounts (Crane, 1979). During the honey ripening process nectars of different sources are mixed. This process can take days to weeks. The ripening process does not only result in a high sugar concentration but also in detectable concentrations of minerals. In honey the maximum concentrations of natural minerals are respectively 1676

µg.g^{-1} K, 76 µg.g^{-1} Na, 35 µg.g^{-1} Mg, 9.4 µg.g^{-1} Fe, 4.09 µg.g^{-1} Mn and 0.56 µg.g^{-1} Cu (Crane, 1979). Plant ashes contain in decreasing order from 10,000 to 0.1 µg.g^{-1} K, Ca, Na, Mg, P, Mn, Zn, Sr, Rb, Ba, B, Cu, Cs, Ti, Pb, Ni, Mo, Li, V, Co and Ag (Lambers et al., 1998).

The estimated amount of food consumed per bee differs per age cohort / age related task. In Table 2, the estimated amounts according to Rortais et al. (2005) are summarized.

Table 2. Sugar and pollen consumption of categories of honeybees

Category of bees	Sugar	pollen
Worker larva	59.4 mg*	5.4 mg
Drone larva	98.2 mg*	no data available
Nurse bees		65 mg
Brood attending bees	272 - 400 mg	
Wax producing bees	108 mg	
Nectar foragers	224 – 898.8 mg	
Pollen foragers	727 – 109.2 mg	
Winter bees	792 mg	

* Larvae are fed with the secretions of the hypopharyngeal glands and mandibular glands produced by the nurse bees, the jelly. Honey / nectar is also added to this menu. The amount of pollen in the larval food is limited; of the total protein need of the larvae less than 5% is provided directly by pollen (Babendreier. 2004).

1.4.9 Nectar and pollen exchange in the colony

Newly collected nectar is distributed among all workers and the larvae, and the major part is stored to be converted into honey (Dadant, 1975; Crane, 1979; DeGrandi-Hoffman & Hagler, 2000; Nixon & Ribbands, 1952). Foraging bees fuel the new foraging flights with newly collected nectar (DeGrandi-Hofmann & Hagler, 2000; Brandstetter, 1988). Nixon & Ribbands (1952) demonstrated with radioactive phosphor spiked sugar fed to six bees in a colony of 24500 bees that as a result of trophallaxis, within four hours 62% of the foraging bees had consumed this sugar. This sugar could be detected in 16 to 21% of the bees of the colony. Within 27 hours, 76% of the foragers and 43 – 60% of all the bees had labelled sugar in the intestines. After 48 hours radioactive sugar could be detected in all larvae. Due to trophallaxis described above almost all bees carry information of the complete colony and not only of a defined forager. The in-hive exchange of pollen is crucial for cross-pollination (DeGrandi-Hoffman & Hagler, 1984).

1.5 State of the art bio-indication with honeybee colonies

Honeybee colonies are recognized as an applicable bio-indicator tool for indication of pollution in the environment. Bio-indication with honeybees and honeybee colonies comprises a broad range. In the scope of this thesis it is restricted to heavy metals, pesticides, polycyclic aromatic hydrocarbons (PAH's) and the impact of metals on bees. The latter is a recent field of study. Bio-indication of plant pathogens is a small field limited to *Erwinia* amylovora. The state of art of this form of bio-indication is referred to in Chapter 6.

Since the beginning of using honeybees for bio-indication, no significant changes have been developed concerning sampling methods. In all recent studies sampling of hive-entering bees is done to collect material on and in bees.

Heavy metals show spatial and temporal variation. Concentrations in bees significantly higher compared to control sites are considered as indications for environmental pollution. Both for heavy metals and PAH's the effect of the landscape on concentrations of metals and PAH's in honeybees is established; bees in open agricultural landscapes have less heavy metals and PAH's compared to bees in urban regions. Unlike heavy metals that are a natural part of pollen and nectar, pesticides are anthropogenic. Foraging bees and pollen appear to be good indicators of pesticides and honey not. Wax accumulated miticides. Miticides applied by the apiculturist to control the varroa mite are frequently detected in the wax. Normally bees die outside the hive. Bee mortality recorded in the vicinity of the hive, (preferably collected in a dead bee trap) exceeding on average 250 bees per week over a monthly recording period, indicate increased mortality due to pesticides (Porrini et al., 2003[a]). This threshold is applied in Italy where frequently monitored sites are installed for bio-indication studies. Heavy metals are a natural part of the honeybee but exposure to increased concentrations can have an impact on the bee's performance as shown for Selenium and Manganese.

To the state of art of applying honeybees for bio-indication new aspects are introduced in this thesis.

1. The honeybee colony including the foraging features of the colony as a Passive Sampling Method (PSM) that samples the environment and that is subsequently subsampled for bio-indication analyses;
2. Introduction of sacrificial and non-sacrificial subsampling of honeybee colonies; applying non sacrificial subsampling means the sampled bees are killed / destructed for analysis; non-sacrificial subsampling is a sampling method by which no bees are taken from the colony and the performance of the colony is not affected;
3. Conflation of environmental technology and apidology;
4. Indications for fine tuning bio-indication studies as there are number of hives to be sampled for representative samples of the environment and indication of thresholds for safe subsampling of hive-entering and in-hive bees;
5. Impact of land use and landscape for bio-indication studies;
6. Early detection of plant pathogens in glass house culture;
7. A national survey of heavy metals.

1.5.1 Heavy metals

The heavy metals Mercury Hg, Chromium Cr, Cadmium Cd and Lead Pb in honeybees in urban sites and wildlife reserves in Central Italy was studied during the period May to October 2007. Twenty-four colonies were sampled monthly by collecting hive entering bees. The metals were analysed for by atomic adsorption measures (atomization temperature 850 ^{o}C, wavelength (nm) Hg 253.7; Cd 228.8; Cr 357.9 and Pb 283.). No Hg was detected. All samples contained Pb, Cr and Cd where Pb showed statistically different concentrations among locations in urban areas and wildlife reserves with the highest concentration near the airport of Rome. July and September were characterized by the highest concentrations of Pb. Also Cd showed spatial and temporal variation and Cr did not (Perugini et al., 2011). The incidence of pesticides and heavy metals over a three-year study (2008-2010) in natural reserves in the Marche region in Italy has been studied in live bees, dead bees and honey. Eleven study sites were set up. On each site two healthy colonies were used. Pesticides were not detected. Heavy metals showed no significant temporal and spatial significant differences in live bees, in dead bees or in honey. Dead bee samples were collected weekly; live bees and honey were sampled monthly from May to October. The threshold for "normal mortality" was set at 250 dead bees per week. Live bees samples consisted of 100 bees. The sampling location was not specified. Heavy metal analyses were done by ICP-AES.

The environmental risk thresholds of heavy metals in honeybees were set for Cd 0.10 mg.kg^{-1}, Cr 0.12 mg.kg^{-1}, Ni 0.30 mg.kg^{-1}, Pb 0.70 mg.kg^{-1}. The most commonly detected heavy metal was Cr. Cr also showed the most frequent increase of the threshold values in live bees and honey. Rainfall lowered the increase of Cr. Cd was detected in live bees but not in honey. Overall in honey, no low concentrations of heavy metals were detected which showed no relationship to the concentrations detected in live honeybees. The results show that live honeybees are the preferred matrix for the detection of heavy metals over dead bees and honey (Ruschioni et al., 2013). Satta et al. (2012) conducted a three-year biomonitoring study in Italy, using honeybees, honey and pollen and ants to detect heavy metals (Cd, Cr and Pb) in a post mining area in Sardinia. Three study sites with three honeybee colonies per site were used. The foragers were sampled from the flight entrance, pollen was collected with a pollen trap and honey was taken from uncapped cells. Additional soil subsamples were taken. Data on heavy metals in forager bees were correlated to soil data. Also pollen provided information on heavy metal contamination, Honey did not. It was concluded that forager bees were efficient environmental pollution bio-indicators. The species variety of ants was lower in polluted sites compared to control sites characterized by lack of vegetation. Lead (Pb) in bees, honey and pollen as sentinels for lead environmental contamination in Western France was studied by Lambert et al. (2012[b]). Sixteen apiaries were used in this study. Forager bees were sampled from the hive entrance. Honey was the least contaminated matrix. Pb concentrations in pollen and bees was about similar (mean bees 0.223 µg.wet g^{-1}; pollen 0.240 µg.wet g^{-1} and showed similar temporal variation. Apiaries in urban and hedgerow landscapes were more contaminated than in cultivated and island landscapes and dry seasons resulted in higher Pb concentrations. Pb was analyzed applying absorption atomic measurement.

1.5.2 Pesticides

Honeybees

Porrini et al. (2003[b]) developed a method to discriminate normal mortality from mortality caused by pesticides. The threshold was set on 250 bees / week / test apiary of two colonies. By data processing with the Index of Environmental Hazard, areas can be characterised with periods of major bee poisoning risk and frequently applied pesticides. The Index was obtained by intersecting mortality with the Index of Pesticide Toxicity (IPT) $\mathrm{IPT} = fcorr \sum_{c=1}^{n} \frac{(ct)c(fp)c}{\mathrm{N}}$

(ct)c = compound toxicity class normalized to the highest value (fp)c = compound persistence factor; fcorr = correction factor; N = number of positive (residue of pesticides) bees.

The threshold of 250 bees is applied in the Italian studies presented below.

The application of using honeybees for bio-indication of pesticides was tested in 2000 by Ghini et al. (2004). In the Bologna region 14 monitoring stations with two honeybee colonies each were installed from April to October 2000. In case mortality exceeded 250 honeybees per apiary, the bees were analyzed. In the 31 samples, 35 pesticides were detected. Organophosphorus pesticides were the most abundant group of pesticides detected. Temporal trends revealed the most incidences occurred in late spring, being associated with the use of pesticides in agricultural areas and less rainfall.

In Italy in 2006, a similar study was done in three monitor stations in the Campanian region. Each hive was provided with a dead bee trap (under basket) to collect dead bees. The threshold for analysis of the dead bees was set at 250 dead bees per monitoring station per week. Chemical analysis of dead bees and palynological analysis of the pollen to determine the crops bees foraged on revealed in 80% (32 sampling dates) at least one pesticide was present. Organophosphorus pesticides were the most frequently detected. Based on the palynological determination of the pollen it was concluded that the bees were exposed due to improper use of the plant protection products, the non-mowing of the native flora and because of spray drift. Additionally, a dioxins analysis was done on one honeybee- and wax sample. In the bee sample dioxins were below the limit of detection. In wax 2.55 mg I-TEF/Kgx10^{-6} residue was detected (Porrini et al., 2014).

The incidence of pesticides and heavy metals over a three-year study (2008-2010) in natural reserves in the Marche region in Italy has been studied in live bees, dead bees and honey. Pesticides have not been detected (Ruschioni et al., 2013).

In the United States a broad survey study of pesticides residues in wax, pollen and bees revealed high levels of multiple pesticides in bee collected pollen. Wax appeared to be the ultimate sink for miticides. In bees the residue concentrations were lower compared to pollen. About 60% of the 259 wax- and 350 pollen samples contained at least one systemic pesticide and 47% had also in-hive applied miticides to control the varroa mite (Mullin et al., 2010).

Honey

The bio-indicator feature of honey to detect pesticides was studied by Balayiannis & Balayiannis (2008). Randomly sampled honey was collected in areas with citrus-, cotton- and sunflower cultures. In 45 out of 50 analysed honey samples pesticides were detected associated with application of the pesticides to the crops mentioned. The analyses revealed also that very often chemicals applied by the apiculturist to control the varroa mites are detectable in honey. The frequency and relative concentrations of pesticides in honeybee colonies in France was studied in the period 2002-2005 (Chauzat et al., 2011). Of the 172 bees samples analysed, 55.7% contained one to five pesticides. Of the 212 pollen samples analysed 69.5 % contained pesticides. Of the 136 honey samples 56.9 % had no pesticide residues. Finally, pesticides were present in 64.9% of wax samples. Pollen loads and wax showed the highest frequency of pesticides and honey the lowest. It was concluded, given the sacrificial feature of honeybee sampling, pollen was the best matrix to detect pesticides in honeybee colonies.

1.5.3 Polycyclic aromatic hydrocarbons (PAH's)

In 2007 Perugini et al. (2009) monitored PAH's in hive entering bees and in honey at eight apiaries. Benzo(a)pyrene was never detected while fluorene, phenanthrene, anthracene, fluoranthene, benz(a)anthracene, benzo(b)fluoranthene and benzo(k)fluoranthene were detected in bees. In honey only phenanthrene, anthracene and chrysene were detected. The PAH's having the lowest molecular weight were dominant in the bees. In honey the concentrations detected were lower compared to bees and did not show any correlation. The applicability of bees, honey and pollen for bio-indication of the PAH's benzo(a)pyrine, benzo(a)anthracene, benzo(b)-fluroranthene and chrysene was studies by Lambert et al. (2012[a]) in 2008 and 2009. Bees were sampled from the hive entrance, honey was extracted from the combs and pollen was collected with a pollen trap. PAHs were analysed with GC-MS/MS measurements. Honey showed the lowest amount of PAH (mean 0.82 $\mu g.kg^{-1}$. In bee samples higher concentrations up to 7.03 $\mu g.kg^{-1}$ were detected and in pollen PAH's were detected in concentrations similar to the ones detected in bees in only one period. The PAH concentrations were significantly influenced by the landscape context; in urban regions with highways and trains more PAH's were detected.

1.5.4 Impact of metals on honeybees

The interest in the impact of metals on bee's performance and health is emerging. Hladum et al. (2012) studied the toxicity of selenium on the honeybee. Honeybees were exposed to selenate (SeO_4^{2-}), the predominant and bioavailable form of Se and to selenomethionine, a naturally occurring Se containing amino acid in plants. Mortality increased in bees exposed to a single dose of 600 µg selenate.ml^{-1} and 6000 µg selenomethionine.ml^{-1}. Chronic exposure via oral feeding concentrations from 60 µg selenate.ml^{-1} and 6000 µg selenomethionine.ml^{-1} resulted in increased mortality. Bees exposed to selenate were less responsive to sucrose stimulation. Gauthier et al. (2016) studied the impact of Al, Pb and Cd on the non-enzymatic anti-oxidant capacity in caged honeybees. Bio-concentration was in the order Cd> Pb> Al. Increasing amounts of Cd resulted in a marked augmentation of MTLP's (metallothionein-like proteins). Pb and Cd increased α-tocopherol. Al altered the lipid peroxidation. Mn negatively affects the foraging ability of the honeybee. Consumption of Mn^{2+} leads to a dose-dependent increase in the brain of octopamine, dopamine and serotonine. The doses tested ranged from 0 to 50 mM Mn. The increase of these biogenetic amines is associated with precocious foraging. Precocious foraging might be associated with decreased navigational ability (Søvik et al., 2015).

1.6 Application of the honeybee colony as Passive Sampling Method (PSM)

Traditionally, a PSM is a tool placed in the aquatic and/or terrestrial environment that passively binds passing material. Depending on the binding matter applied in the passive sampler, this binding can be selective or general. Passive samplers integrate spatial and temporal surveys, have low costs, do not require a power supply, have flexibility of deployments and use, can be used anywhere and in large numbers and have low operational costs and require no specialist training for (re)placements. The disadvantage compared to active spot check sampling is that the result of passive sampling is qualitative or semi-quantitative. The amount of vector material like air (m^3) or water (m^3) is not measured whereas in active sampling this is a known term. There are two types of mechanical PSMs: 1) partition samplers, also named equilibrium samplers in which the contaminant will dissolve and in time will reach equilibrium with the environment and 2) adsorption samplers at which the matter will adsorb through chemical or physical surface binding. Assuming the adsorption surface is sufficient, the adsorption will be more

or less linear and no equilibrium will be reached (Tang et al., 2001; Targa & Loader, 2008; STOWA, 2014).
The honeybee colony can be added as an adsorption sampler to the concept of Passive Sampling Methods. The honeybee unintentionally, passively samples the environment by collecting and accumulating pollutant and pathogenic matter along with food collection, indicating the quality of the environment.

1.6.1 Bio-sampling and sample processing by the honeybee colony

Foragers of the honeybee colony scavenge the environment in their search for food: nectar, pollen and water. Nectar is collected from flowers and extra floral nectaries. Honeydew (sweet excretion of aphids) is collected from plant leaves and coniferous needles. Pollen is collected from flowers. Water is collected from flowers, on leaves (guttation), in puddles and ponds. Part of the water is collected in the hive as condensate formed from the honey ripening process. Propolis (resin) used for hygienic purposes is collected from buds. The result of the forager trips of the complete forager cohort is accumulated in the hive. During food collection, atmospheric deposition of small particles in flowers, possibly containing heavy metals, POPs, radioactive matter and epi- and endo plant pathogens are collected unintentionally. The nectar collecting forager lands on the flower and finds her way via the petal surface to the nectaries. The forager collects pollen by pulling or shaking the grains present in the anthers and on the petals, onto their body. Subsequent, during pollen collection and during the return flight, the head and the front part thorax are brushed with the forelegs. The backside of the thorax is brushed with the middle legs. The pollen grains are collected in the metatarsal brushes of the middle leg pair and transferred to the brushes on the metatarsi of the hind legs. From these brushes the grains are combed in the stiff hair row on the tibia of the hind leg and pressed (Hodges, 1974). Honeybees are unable to clean themselves completely; mainly behind the head, the central dorsal parts of the first thorax segments and the first two abdomen segments, particles are left (Free & Williams, 1972; Lukoschus, 1957). Moreover, some of the particles get stuck between the branched hairs of the honeybee (Wadl et al., 2009). As a result of this incomplete cleaning and subsequent in-hive physical contact, within 3 to 4 hours 95 to 100 % of the in-hive bees have pollen and particles in the hairs from other bees (DeGrandi-Hoffman et al., 1986). Bees that have never left the colony have relatively more small pollen grains in the hair (Paalhaar et al., 2008). Each bee that starts the foraging trip has 4000 – 13000 pollen grains in its fury hairs (Free & Williams, 1972). The pollen forager collects per collection flight 12 – 22 mg pollen (Maurizio, 1953). The estimated average weight of a pollen

grain is 50 to 100 ng (Kleinjans et al., 2012; Babendreier et al., 2004). Based on weight, between 150,000 and 300,000 grains are collected. After self-grooming, 2 to 4% is left on the honeybee's body. Nectar foragers collect fluid and will passively scavenge particles of the flower surface. Brushing the fury hairs to translocate particles into the corbicula is not or not done as frequently by the nectar collectors as it is by the pollen foragers (Westerkamp, 1991). The active collection of nectar and pollen and the passively scavenging of non-pollen particles in the flowers and on petals and leaves makes the individual honeybee a micro-sampler and bio-indication tool. The transport to and the accumulation of all matter collected in the hive and the in-hive physical exchange of particles makes the application of the honeybee colony for bio-indication a passive sampling method.

1.6.2 Subsampling of the honeybee colony

Unlike the mechanical PSM's which are analysed as a complete device, the PSM honeybee colony must be subsampled to obtain material for analysis (Figure I). Subsampling honeybees from the honeybee colony, both sacrificial and non-sacrificial, is an integral part of the PSM honeybee colony. The wording sacrificial- and non-sacrificial subsampling is new. Sacrificial subsampling means that the bees or honeybee colony's products are sacrificed / destroyed for analysis. Per definition non-sacrificial subsampling does not affect the honeybee colony. By applying non-sacrificial subsampling, neither the number of bees of the colony nor the colony's development and behaviour are affected. The bees stay alive and the bee's products intact. In general, a distinct practical pro of non-sacrificial subsampling is that it can be conducted by non-professional beekeepers and therefore can be applied everywhere apiculture is practiced. Non-sacrificial subsampling of a honeybee colony has an ethical aspect; killing living organisms should in my opinion be restricted to cases where the sampling cannot be done without it.

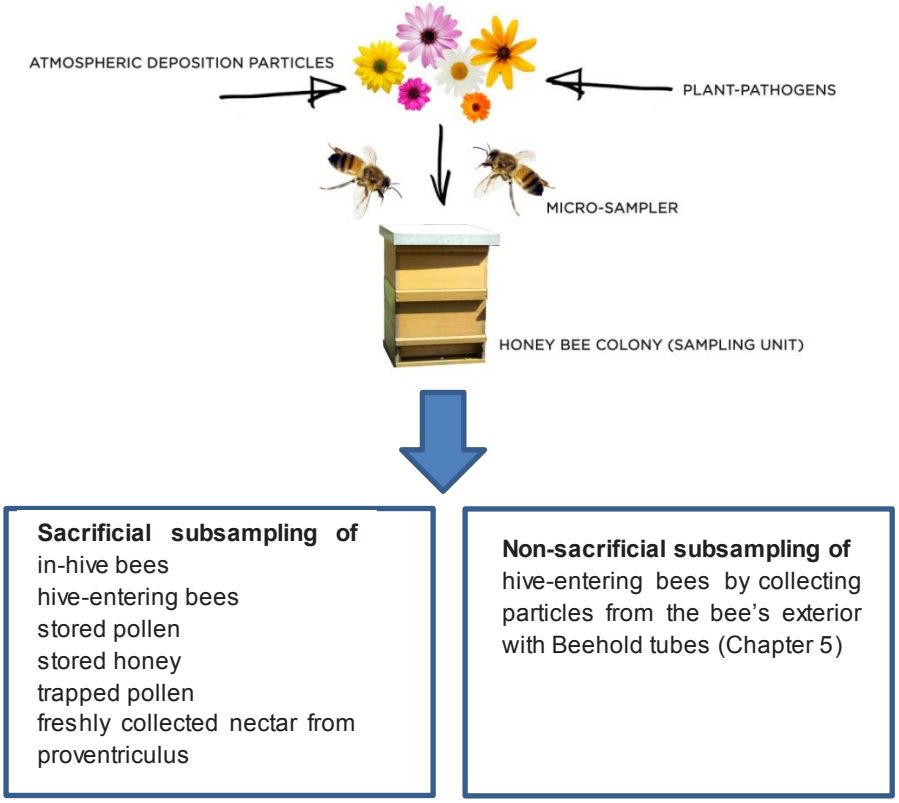

Figure IV. Schematic overview of the PSM honeybee colony and subsequent subsampling

Subsampling honeybees from a colony

The objective of a bio-indication study is to record the qualitative presence / absence of target matter. Semi-quantitative results can only be indicated in cases where the presence of target matter in the subsample exceeds the control values significantly. Subsampling the honeybee colony requires a calculated sample size, big enough to have a reasonable chance to detect target matter e.g. metal containing atmospheric deposition of combustion and traffic, metal containing PM, metal containing road dust, POPs from soil erosion and airborne epi-plant plants pathogens and endo-plant pathogens in the environment. Depending on the objective of the study, three subsampling methods to be applied under the precondition of a correct sample size and sample composition are: 1) sacrificial subsampling of hive-entering bees; 2) sacrificial subsampling of in-hive bees; 3) non-scarified subsampling of hive-entering bees.

Sample size and sample composition

The sample size depends on the proportion of bees carrying target matter and the probability of detection. With the binomial probability theory equation $N = \ln(1-D)\,/\ln(1-P)$ the sample size can be calculated for hypothetic proportions of bees carrying target matter. *N* = sample size, ln = natural logarithm, *D* = probability (power) of detection, *P* = minimal proportion of bees carrying target matter which can be detected with the required power (Pirk et al., 2013). The precondition is that bees in the sample carry sufficient matter to be detectable which depends on the Limit of Detection (LOD) of the analysis method. In Figure V, the relation between minimal sample size and proportion bees carrying target matter is delineated. The sample size increases significantly in case about < 10% of the bees in the sample carry target matter. For rare target matter up to several hundreds and for abundant target matter tens bees should be sampled. In case it takes more bees for a LOD, the sample size must be customized. For example if it takes 10 bees to have a detectable amount target matter, the sample size calculated should be multiplied with a factor 10.

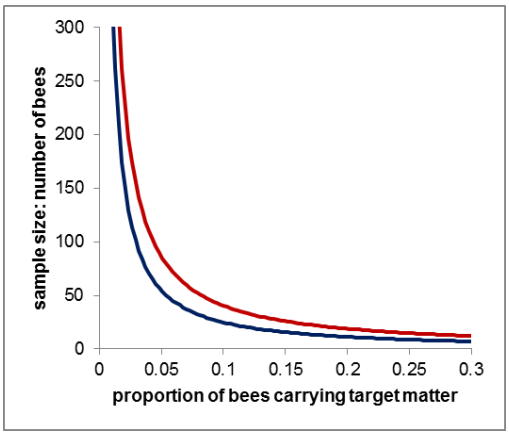

Figure V. Relation between proportion of bees carrying target matter and sample size.
In case 1% of the bees carry target matter, the sample size should be minimally 298 (P=0.95) and 458 (P = 0.99) to have at least one bee carrying target matter.
In case 5% of the bees carry target matter, the sample size should be minimally 44 (P=0.95) and 90 (P = 0.99) to have at least one bee carrying target matter.
In case 25% of the bees carry target matter, the sample size should be minimally 10 (P=0.95) and 16 (P = 0.99) to have at least one bee carrying target matter.

Sacrificial subsampling of in-hive bees

Pertaining to bio-indication and detecting target matter, the pro of sampling in-hive bees is that, due to trophallaxis and in-hive physical exchange, the majority of the bees will carry target matter within hours to days (paragraph 1.4). It is obvious that amounts per bee depend on the influx of target matter and colony size. Sampling the bees from defined locations in the hive provides information of the age cohort. On brood

frames all age cohorts are present and the very young bees (days) are overrepresented. Taking bees from the first bee-lane between the outer brood frame and the first frame without brood, results in a mixture of bees of all age classes. This sample is sort of homogeneously but will change during the course of the bee season as the composition of the age classes of bee's changes in time. At the top of the hive, where the honey is stored, all age cohorts are present but forager bees are dominant (Steen et al., 2012[b]). Forager bees that visited flowers contaminated with target matter carry more target matter than in-hive bees because of physical dilution of target matter inside the hive. On the other hand, in case of constant influx of target matter, in-hive bees can accumulate considerable amounts of target matter. Applying sacrificial subsampling and processing the complete bee results in detecting target matter both in and on the bee. Sacrificial subsample processing by rinsing the bee to remove matter from the exterior of the bee, applying detergent like Tween 80 or Triton X, facilitates the removal but also kills the bee.

Sacrificial subsampling of hive-entering bees

The pro of taking bees from the hive entrance is that the forager bees will have relatively higher amounts of target matter compared to in-hive bees, especially at the start of the influx of target matter. The con of subsampling bees from the hive entrance is a less consistent composition of the sample in the term of age cohorts. Although the majority of the bees entering the hive are foragers bringing in nectar, pollen and water, the composition the bee cohort on the flight entrance alters frequently. The ratio pollen foragers, nectar foragers and water collectors depends on the colony demand and the availability of the food sources (par. 1.4). Additionally, the ratio of pollen foragers, nectar foragers and bees making orientation flights can change within minutes. Observation of the hive entrance, every beekeeper will confirm this, show periods of frequent pollen inflow, periods of non-pollen inflow and periods with a lot of what beekeepers call playing bees in front of the hive, the orienting bees. Above this, there is variation of flight frequency of a colony during the day and between colonies in the apiary. Based on the annual nectar and pollen needs of the colony of respectively 125 and 25 kg and the fact that the weight of the collected nectar and pollen is about the same, five times more nectar collectors will enter the hive. Nectar collectors comprise the largest cohort for scavenging the flowers / environment. The water collecting cohort is not included as part of the water is collected in-hive.

Non-sacrificial subsampling of bees from a honeybee colony
Collecting target matter from the exterior of the honeybee without killing her, is relatively new. In an *Erwinia amylovora* study in Austria conducted in the period 2012 - 2014, plastic sheets were used. Both in- and outgoing bees were forced to walk over this sheet to trap the bacterium. This has been done successfully, *E. amylovora* was detectable on the transparent cover sheets provided to colonies in orchards where this bacterium was present (Halbwirth et al., 2014). I modified this concept and developed the Beehold device. The modifications are: splitting the in- and outgoing bees, adding a bee counter of the hive entering bees and putting a moderately sticky polyethylene glycol layer on plastic sheets via which hive-entering bees must pass to enter the hive. The detailed description of the Beehold device is in Chapter 5.

Sacrificial sampling of bee's products
Sampling honeybees in bio-indication study of the heavy metals Cd and Pb give more reliable results than sampling wax, pollen, propolis and honey (Conti & Botre, 2001). The focus of PSM honeybee colony as presented and discussed is mostly on honeybees and not on bee products.

Stored pollen / beebread
Collected pollen is stored in cells and covered with a layer of honey. This creates an anaerobic condition in the cell. Via a microbiological silage process in which lactic acid bacteria are involved, the pollen turns into beebread (Vásquez & Olofsson, 2009). Stored pollen may provide information of target matter. It is a distinct pro that target matter and botanical origin of the pollen can be combined directly. There is a temporal aspect to this sampling. Pollen collection and subsequent beebread consumption depends on the number of brood cells in the colony. Unlike honey, no overwintering surplus of pollen is stored in the colony. Pollen is stored for a short period and collected more or less on demand of the colony. Therefore, there is no specific turn-over period of pollen in the colony and there is no pollen archive in the hive. The turnover period of beebread varies from days to months. Pollen collected in summer will be consumed rapidly and pollen collected in late summer / autumn can be found for a longer period in the hive. Due to this process the bioavailability of contaminants on pollen may change. To my knowledge there are no studies about the effect of silage of beebread on the bioavailability of contaminants.

Honey
Honey is not an appropriate indicator of environmental pollution. It is the mixture of various nectar sources mixed during the honey making process, a temporal process. Additionally, bees filtering the nectar in the honey sac (proventriculus) directly after collection bringing most of the particles like pollen, combustion particles, particulate matter (PM) and micro-organisms sucked in along with nectar from the nectar, into the alimentary tract (Kellner, 1981). Additionally, due to semi-undirected foraging area of honeybee colonies it is inaccurate to correlate origins and concentrations of pollutants to specific areas. Although to this statement must be added that the feature of honeybee colonies to forage as close by the hive as possible, provided that food sources are available, in combination with pollen determination, the foraging area can be indicated.

Trapped pollen
Pollen can be a preferred subsample for the determination of plant pathogens associated with pollen and of pesticides. An example is the Blueberry shock ilarvirus. This virus is present on and in pollen of the highbush blueberry (*Vaccinium corymbosum* L.). In a honeybee colony the virus remains infectious for minimally one week (Bristow & Martin, 1999). To bio-indicate residues of pesticides subsampling of pollen is a distinct method. There are many studies in which this subsampling is applied. I mention two exemplary surveillance studies. In France a three-year field survey demonstrated residues of pesticides in 0 – 50% of the pollen collected (Chauzat et al., 2006). In the USA a significant part of collected pollen in apiaries in Florida and California (2007-2008) contained residues of pesticides (Mullin et al., 2010).

Recent collected nectar from the proventriculus of hive-entering bees
To sample nectar, hive-entering bees can be dissected to remove the crop (Reetz & Wallner, 2014). Another sacrificial method is to make the bees throw up the collected nectar by pressing the abdomen (Gary & Lorenzen, 1979).

Non-sacrificial subsampling of bee's products
Pollen samples of trapped pollen or of ensilaged pollen stores in the cells (beebread), if taken in relatively small amounts related to the influx, can be done without harming the colony and the colony's development. Bees will fill in the gaps by extra foraging if needed.

1.7 Framework of the application of the honeybee colony for bio-indication

Based on the honeybee colony's foraging features, in-hive conditions and target matter, seven steps, delineated in Figure IV, apply for the passive sampling method: honeybee colony. The target matter (1) and where this might be present (2) determines where to locate the PSM honeybee colony (3). After the honeybee colony has scavenged the environment it must be decides to subsample individual colonies of an apiary or take pooled subsamples of the apiary (4) and how the colony can be subsampled (5). Depending on the target matter, colony strength and the proportion of bees possibly carrying target matter sacrificial, non-sacrificial or a combination of both sampling methods can be applied (6). The final step is the analysis of the target matter (7). This 7-step flow chart and its practical implications will be discussed in paragraph 8.2.

Framework for the application of PSM honeybee colonies from target matter to analysis.

1. Study objective (target matter) e.g. heavy metals, POPs, plant-pathogens collected by the forager bee and accumulated in the hive (par. 1.2: Bio-indication, definitions & brief historical overview; 1.5 State of the art honeybee colony of bio-indication);
2. Target matter location (Par.1.3: Environmental pollution and plant diseases, Source-Path-Receptor approach);
3. Location the honeybee colony is located for its application as Passive Sampling Method (Par 1.4.2 Features honeybee & honeybee colony as PSM);
4. Choice, depending on the study objective to sample individual colonies or take a pooled sample of the apiary (more than one colony on the same location (Par 1.4 Features honeybee & honeybee colony as PSM).
5. Subsampling location (1.4 Features of the honeybee & honeybee colony PSM; 1.6 sacrificial and non-sacrificial subsampling of the PSM honeybee colony;

6.a/b Sacrificial and non-sacrificial subsampling (Par 1.6: sacrificial and non-sacrificial subsampling of the honeybee colony);

7. Subsample analysis of the processed bee or bee's products.

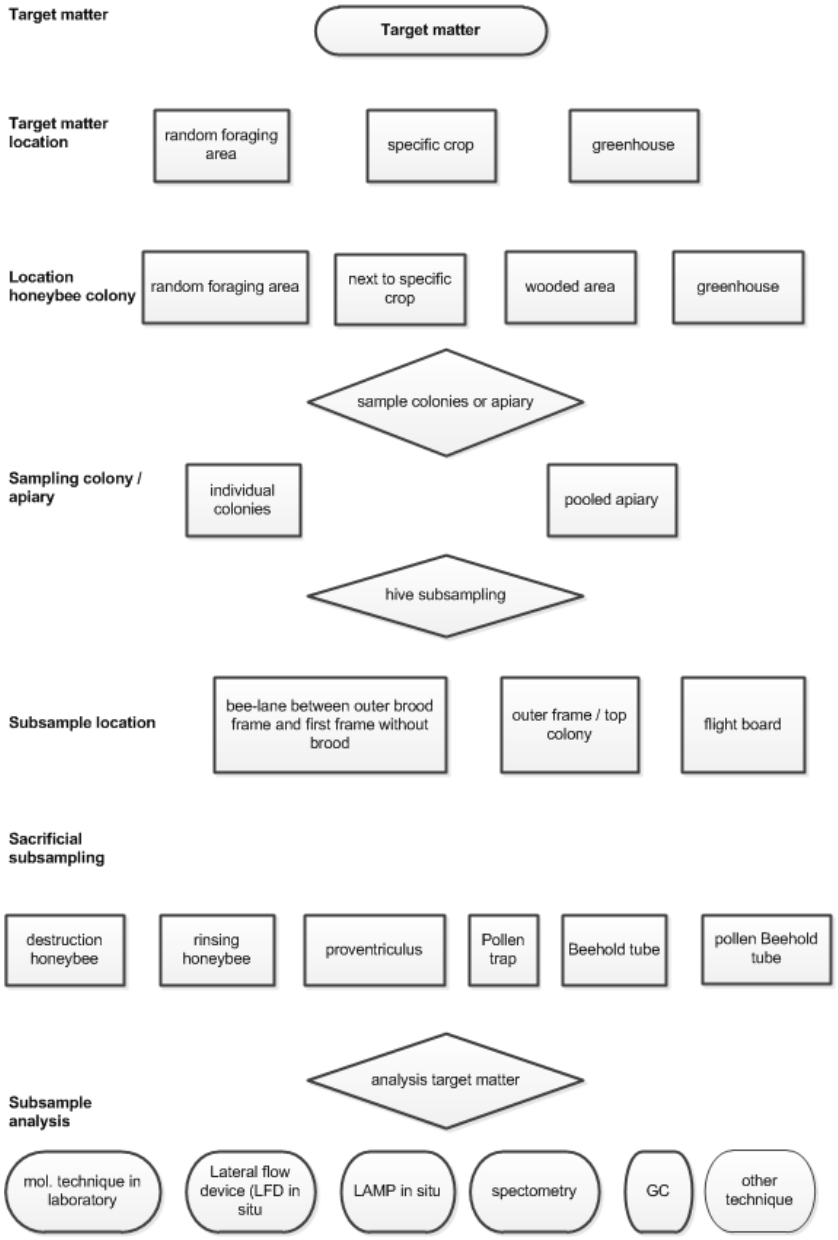

Figure IV. Flow chart 7-steps frame work

Chapter 2

Spatial and temporal variation of metal concentrations in adult honeybees (*Apis mellifera* L)

J.J.M. van der Steen; J. de Kraker and J.T.C. Grotenhuis 2012. *Environmental Monitoring and Assessment* 184: 4119-4126 doi 10.1007/S10661-011-2248-7

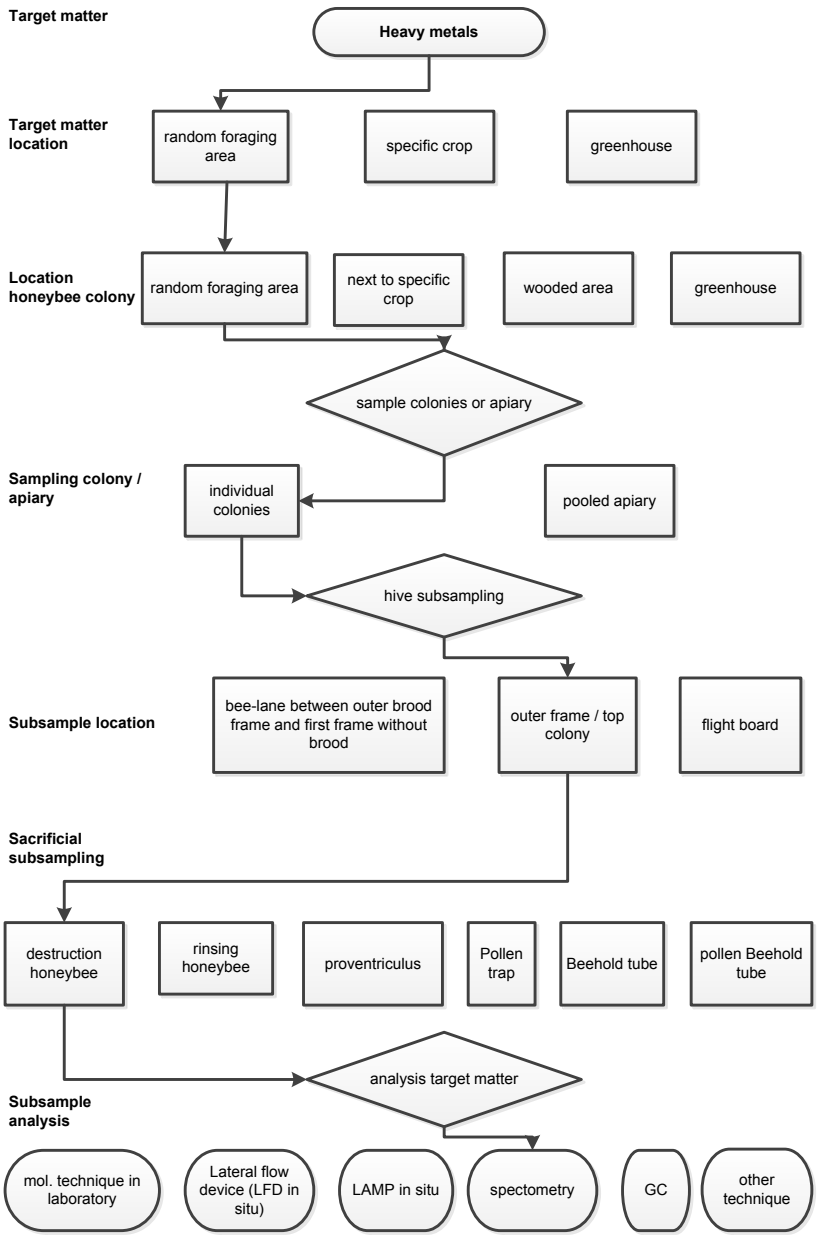

Figure I. Bio-indication flow chart: Spatial and temporal variations of metal concentrations in adult honeybees (*Apis mellifera* L)

Abstract
Honeybees (*Apis mellifera* L) have great potential for the detection and monitoring of environmental pollution, given their wide-ranging foraging behavior. Previous studies have demonstrated that concentrations of metals in adult honeybees were significantly higher at polluted than at control locations. These studies focused at a limited range of heavy metals and highly contrasting locations, and sampling was rarely repeated over a prolonged period. In our study, the potential of honeybees to detect and monitor metal pollution was further explored by measuring the concentration in adult honeybees of a wide range of trace metals, 9 of which were not studied before, at three locations in the Netherlands over a 3-month period. The specific objective of the study was to assess the spatial and temporal variation in concentration in adult honeybees of Al, As, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, V and Zn. In the period of July-September 2006, replicated samples were taken at 2-week intervals from commercial-type bee hives. The metal concentration in µg per gram honeybee was determined by Inductive Coupled Plasma – Atomic Emission Spectrometry (ICP-AES). Significant differences in concentration between sampling dates per location were found for Al, Cd, Co, Cr, Cu, Mn Sr, Ti and V, and significant differences in average concentration between locations were found for Co, Sr and V. The results indicate that honeybees can serve to detect temporal and spatial patterns in environmental metal concentrations, even at relatively low levels of pollution.

2.1 Introduction

Bio-indication is a time dependent, sensitive registration of anthropogenic or anthropogenically altered environmental factors, by distinguished dimensions of biological objects and biological systems under defined circumstances (Stöcker, 1980). Honeybees (*Apis mellifera* L) are potentially highly useful as bio-indicators for the detection and monitoring of environmental pollution, given their worldwide usage for honey production and pollination and their wide-ranging foraging behavior (Bromenshenk & Preston, 1986; Raeymaekers, 2006). Not surprisingly, studies on the use of honeybees and bee products for environmental monitoring have a relatively long history, dating back to at least 1935 (Crane, 1984). Environmental pollutants included in these studies were, among others, pesticides, radioactive elements and heavy metals (Devillers & Pham-Delègue, 2002). As for the latter, honeybees may take up heavy metals from all environmental compartments: soil, vegetation, air and water (Bromenshenk et al., 1985; Porrini et al., 2003[b]). Heavy metals end up in these compartments after emission from a variety of mainly anthropogenic sources. A major source of heavy metals in the atmosphere, for example, is the combustion of fossil fuels which results in the emission of ultrafine metal-containing particles. These airborne particles eventually deposit on vegetation, soil or surface water. Honeybees pick up heavy metals from the environment through a wide range of pathways: by ingestion of polluted surface water, pollen and nectar, by impaction and inhalation of particles during flight, and by adhesion of particles to their hairy bodies when moving over plant and soil surfaces during foraging. In this way, honeybees provide an integrated sample of the environmental compartments in the area within their flight range (c. 7 km^2, Bromenshenk et al., 1985), and can therefore serve to indicate anomalies in the environmental distribution of trace metals in time and space (Raeymaekers, 2006). Possible mechanisms behind detected anomalies can then be studied with other, more specific methods.

Heavy metals in bees and in bee products have been the subject of many studies (e.g., Bromenshenk et al., 1985; Conti & Botré, 2001; Fakhimzadeh & Lodenius, 2000; Kalnins & Detroy, 1984; Leita et al., 1996; Roman, 2005; Veleminsky et al., 1990). The most frequently studied metals were lead, cadmium, chromium, copper and zinc, which are known pollutants from transport and industrial activity, disseminated via combustion gasses. Most studies focused on a limited number of metals and highly contrasting locations, and sampling was rarely repeated over a prolonged period. In our study, the potential of honeybees to detect and monitor metal pollution was further explored by measuring the

concentration in adult honeybees of a wide range of 18 trace metals, 9 of had not been studied before, at three locations over a 3-month period. The specific objective of the study was to assess the spatial and temporal variation in concentration in adult honeybees of aluminium (Al), arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lithium (Li), manganese (Mn), molybdene (Mo), nickel (Ni), lead (Pb), antimony (Sb), selenium (Se), tin (Sn), strontium (Sr), titanium (Ti), vanadium (V) and zinc (Zn). We chose to study the metal concentrations in adult worker bees, as these are considered to provide more sensitive, reliable and up-to-date information about exposure of bees to metals in the environment than concentrations in pollen and honey (Bromenshenk et al., 1985; Jones, 1987; Fakhimzadeh & Lodenius, 2000; Porrini et al., 2002[a]; Veleminsky et al., 1990).

2.2 Materials and Methods

The bio-indication scheme is presented in the flow chart on page 46 (Figure I).
At three locations in the Netherlands, three honeybee colonies (replicates) per location were placed. During a three-month period, from July to September 2006, samples of each honeybee colony were taken every 14 days. The concentration of metals in honeybees was determined chemically.

2.2.1 Study locations

Location Maastricht, Limburglaan. Maastricht is an urban area with cement industry and glass industry, and is located close to large industrial areas such as Liège in Belgium. The honeybee colonies were placed near the city centre, on the roof of the provincial government building.
Location Buggenum, Dorpstraat. Buggenum is a village in a rural area about 60 kilometres north of Maastricht. In Buggenum bricks are produced and a large electric power plant is situated next to the village. This plant is powered by coal, natural gas and biomass.
Location Hoek van Holland, Prins Hendrikstraat. Hoek van Holland is situated in the Rijnmond region at the river-mouth of the Nieuwe Waterweg, at the North Sea coast. The Rijnmond region includes the port of Rotterdam and a large industrial area where among others petrochemical industry, tank storage and tank transfer and waste treatment plants are situated.

2.2.2 Honeybee sampling method

Honeybee colonies were kept from winter until summer in the same apiary in Wageningen (The Netherlands) after which they were distributed between the three locations. Three honeybee colonies (*Apis mellifera* L) were placed at each location. The colonies were kept in one storey wooden hives with ten frames (Simplex measures NEN 061-50). This is the most commonly, commercially used type of hive in the Netherlands. During the study period of July, August and September 2006, every two weeks a random sample of 100 to 150 worker honeybees was taken from the outer frame of the hive that was occupied with bees but without brood. Sampling was done by brushing bees with a plastic brush into a plastic container. This resulted in 18 bee samples per location (three replicates of six sampling dates) to be analyzed for all metals per location. The samples were transported in a cooler box and stored in the freezer at -20 ± 5 ^{0}C until analysis.

2.2.3 Measurement of heavy metal concentrations in bees

The chemical analyses on metals were carried out by the environmental research laboratory of the Province of Limburg (Hoofdgroep Milieu en Water, Bureau onderzoek en advies), using the Inductive Coupled Plasma – Atomic Emission Spectrometry (ICP-AES) technique. From each sample (i.e., from each combination of colony, sampling date and location), 25 frozen worker bees were taken at random from the sample. The bees were subsequently weighed, dried during 24 hours at 120 ^{o}C, weighed, and destructed by boiling the sample at 170 °C in a mixture of 25 ml HNO_3 (70%) and HCl (37%) at a ratio of 1 to 3 (aqua regia). The resulting liquid was topped up to 50 ml with demi-water. Five ml of the 50 ml was filtered through a cotton wool filter and analyzed using ICP-AES. The resulting signals (ng ml^{-1}) were converted to ng g^{-1} (ppb) bee with a conversion factor (volume sample / (weight bees x mean percentage dry weight)) resulting in ppb metal dry weight which was subsequently converted to µg per gram bee (µg g^{-1} dry material (dm)). The overall weight loss of the bee samples as a result of the drying process was 68%.

2.2.4 Statistical analyses

Per metal, a GLMM (generalized linear mixed model) analysis was done assuming a lognormal distribution of the concentration data. Differences in concentrations between dates or locations were considered significant at P values ≤ 0.05, using Tukey's Multiple Comparisons test. Temporal differences in concentration were assessed by comparing the 2-weekly sample values for each location. Spatial differences in concentration were assessed by comparing the mean values of the entire 3-month study period between locations.

2.3 Results and discussion

2.3.1 Temporal and spatial variation in metal concentrations

The 2-weekly sample values of metal concentrations (averages of the three replicate samples) are presented for each location in Table 1. Different superscript letters indicate per location (row) statistically significant differences between metal concentrations in samples taken at different dates. For nine of the metals included in our study no differences between 2-weekly sample values were found, and the concentrations were apparently constant over time. For the other nine metals (Al, Cd, Co, Cr, Cu, Mn, Sr, Ti and V) significant differences between 2-weekly sample values were found at at least one of the study locations. The fluctuations in concentration indicate a significant variation in exposure of honeybees to these metals in the environment.

For all but three metals, no significant differences in mean concentration (over the entire study period) between locations could be detected. This indicates that the overall environmental exposure of honeybees to the metals Al, As, Cd, Cr, Cu, Li, Mn, Ni, Pb, Sb, Se, Sn, Ti and Zn during the study period was comparable in Maastricht, Buggenum and Hoek van Holland. The overall mean concentrations of Co, Sr and V, however, differed significantly between the study locations (Table 2). These spatial differences might be caused by differences in industrial activity near these locations. Markedly more significant temporal (nine) than spatial (3) differences were found. This probably indicates that the temporal fluctuations in source strength over a 3-month period are greater that the more structural differences between locations. In a small and densely populated country as the Netherlands, spatial differences may be expected to be limited. However, as our method does not provide information on sources and mechanisms, any explanation of the observed differences, spatial as well as temporal, will remain speculative. In case the variations in metal concentrations in time and space detected with honeybees are considered to be a reason of concern, other, more specific methods will have to be used to investigate the causal mechanisms. For example, use could be made of the Enrichment Factor (Chester et al., 1999), to determine whether trace metals in the air have significant non-crustal sources.

Table 1. Metal concentrations in worker honeybees (µg g^{-1} dm) from three locations, sampled at 2-weekly intervals (July-September 2006). Concentration values are calculated as means of three independent replicate samples.

Element	Location	Sampling date					
		20 July	3 Aug	17 Aug	31 Aug	14 Sept	28 Sept
Al	Maastricht	15.10^{b}	10.75^{ab}	5.9^{a}	6.8^{a}	9.89^{ab}	9.3^{ab}
Al	Buggenum	11.55^{bc}	10.93^{bc}	4.6^{a}	6.6^{ab}	15.52^{c}	11.07^{bc}
Al	Hoek van Holland	10.70^{bc}	13.20^{c}	6.15^{ab}	5.57^{a}	12.17^{c}	9.49^{abc}
As	Maastricht	0.72^{a}	0.70^{a}	0.66^{a}	0.76^{a}	0.68^{a}	0.83^{a}
As	Buggenum	0.70^{a}	0.69^{a}	0.77^{a}	0.76^{a}	0.70^{a}	0.71^{a}
As	Hoek van Holland	0.68^{a}	0.67^{a}	0.69^{a}	0.69^{a}	0.69^{a}	0.75^{a}
Cd	Maastricht	0.09^{ab}	0.09^{ab}	0.07^{a}	0.17^{ab}	0.24^{ab}	0.75^{b}
Cd	Buggenum	0.14^{a}	0.25^{a}	0.10^{a}	0.18^{a}	0.19^{a}	0.71^{a}
Cd	Hoek van Holland	0.13^{ab}	0.06^{ab}	0.05^{a}	0.05^{a}	0.50^{b}	0.25^{ab}
Co	Maastricht	0.10^{a}	0.10^{a}	0.08^{a}	0.11^{a}	0.14^{a}	0.12^{a}
Co	Buggenum	0.26^{ab}	0.21^{ab}	0.16^{a}	0.33^{b}	0.16^{a}	0.16^{a}
Co	Hoek van Holland	0.10^{a}	0.11^{a}	0.09^{a}	0.10^{a}	0.11^{a}	0.09^{a}
Cr	Maastricht	0.27^{b}	0.21^{ab}	0.16^{a}	0.18^{ab}	0.24^{ab}	0.23^{ab}
Cr	Buggenum	0.23^{ab}	0.23^{ab}	0.15^{a}	0.21^{ab}	0.25^{ab}	0.28^{b}
Cr	Hoek van Holland	0.27^{ab}	0.22^{ab}	0.18^{a}	0.18^{a}	0.28^{b}	0.22^{ab}
Cu	Maastricht	14.69^{a}	18.37^{a}	19.16^{a}	16.86^{a}	17.64^{a}	19.74^{a}
Cu	Buggenum	12.69^{ab}	11.65^{a}	11.85^{a}	15.50^{ab}	12.57^{ab}	19.77^{b}
Cu	Hoek van Holland	14.21^{a}	14.33^{a}	12.84^{a}	13.13^{a}	15.23^{a}	15.80^{a}
Li	Maastricht	0.05^{a}	0.02^{a}	0.01^{a}	0.01^{a}	0.02^{a}	0.02^{a}
Li	Buggenum	0.03^{a}	0.02^{a}	0.02^{a}	0.01^{a}	0.02^{a}	0.03^{a}
Li	Hoek van Holland	0.05^{a}	0.04^{a}	0.01^{a}	0.01^{a}	0.03^{a}	0.01^{a}
Mn	Maastricht	24.45^{ab}	28.31^{ab}	20.69^{a}	41.98^{ab}	68.76^{b}	45.10^{ab}
Mn	Buggenum	31.04^{a}	28.42^{a}	29.16^{a}	47.3^{4a}	48.40^{a}	50.80^{a}
Mn	Hoek van Holland	32.11^{a}	30.44^{a}	26.48^{a}	28.87^{a}	34.48^{a}	34.37^{a}
Mo	Maastricht	0.77^{a}	1.16^{a}	1.07^{a}	0.64^{a}	0.73^{a}	0.54^{a}
Mo	Buggenum	0.53^{a}	0.42^{a}	0.36^{a}	0.57^{a}	0.75^{a}	0.66^{a}
Mo	Hoek van Holland	0.55^{a}	0.55^{a}	0.51^{a}	0.50^{a}	0.68^{a}	0.46^{a}
Ni	Maastricht	0.37^{a}	0.44^{a}	0.34^{a}	0.26^{a}	0.22^{a}	0.19^{a}
Ni	Buggenum	0.29^{a}	0.47^{a}	0.25^{a}	0.28^{a}	0.29^{a}	0.29^{a}
Ni	Hoek van Holland	0.43^{a}	0.35^{a}	0.29^{a}	0.26^{a}	0.41^{a}	0.20^{a}
Pb	Maastricht	0.41^{a}	0.37^{a}	0.26^{a}	0.31^{a}	0.55^{a}	1.26^{a}
Pb	Buggenum	0.27^{a}	1.10^{a}	0.19^{a}	0.30^{a}	0.53^{a}	0.58^{a}
Pb	Hoek van Holland	1.00^{a}	0.30^{a}	0.27^{a}	0.35^{a}	1.67^{a}	0.55^{a}
Sb	Maastricht	0.12^{a}	0.10^{a}	0.11^{a}	0.11^{a}	0.18^{a}	0.13^{a}
Sb	Buggenum	0.11^{a}	0.10^{a}	0.12^{a}	0.15^{a}	0.09^{a}	0.12^{a}
Sb	Hoek van Holland	0.19^{a}	0.07^{a}	0.11^{a}	0.09^{a}	0.10^{a}	0.11^{a}

Element	Location	Sampling date					
		20 July	3 Aug	17 Aug	31 Aug	14 Sept	28 Sept
Se	Maastricht	1.38[a]	1.23[a]	1.24[a]	1.30[a]	1.50[a]	1.53[a]
Se	Buggenum	1.35[a]	1.27[a]	1.24[a]	1.38[a]	1.28[a]	1.22[a]
Se	Hoek van Holland	1.24[a]	1.20[a]	1.21[a]	1.17[a]	1.15[a]	1.17[a]
Sn	Maastricht	0.51[a]	0.44[a]	0.47[a]	0.44[a]	0.62[a]	0.52[a]
Sn	Buggenum	0.54[a]	0.68[a]	0.49[a]	0.43[a]	0.50[a]	0.42[a]
Sn	Hoek van Holland	0.76[a]	0.47[a]	0.51[a]	0.47[a]	0.44[a]	0.44[a]
Sr	Maastricht	1.82[ab]	2.99[b]	1.54[ab]	0.95[a]	1.05[a]	1.00[a]
Sr	Buggenum	0.99[a]	1.02[a]	0.70[a]	0.86[a]	0.89[a]	1.00[a]
Sr	Hoek van Holland	2.18[a]	2.40[a]	1.97[a]	1.36[a]	1.33[a]	0.94[a]
Ti	Maastricht	0.45[b]	0.37[ab]	0.16[a]	0.22[ab]	0.43[b]	0.47[b]
Ti	Buggenum	0.34[bc]	0.41[bc]	0.09[a]	0.17[ab]	0.55[c]	0.39[bc]
Ti	Hoek van Holland	0.54[c]	0.51b[c]	0.20[ab]	0.17[a]	0.50[bc]	0.35[abc]
V	Maastricht	0.040[ab]	0.032[ab]	0.015[a]	0.015[a]	0.054[b]	0.033[ab]
V	Buggenum	0.028[b]	0.026[b]	0.006[a]	0.006[a]	0.042[b]	0.029[b]
V	Hoek van Holland	0.083[a]	0.14[b]	0.10[a]	0.093[a]	0.31[b]	0.31[b]
Zn	Maastricht	67.81[a]	72.36[a]	59.18[a]	72.03[a]	82.83[a]	100.46[a]
Zn	Buggenum	73.66[a]	75.54[a]	70.70[a]	94.52[a]	71.60[a]	95.44[a]
Zn	Hoek van Holland	63.38[a]	68.98[a]	61.61[a]	61.14[a]	71.49[a]	74.76[a]

Table 2. Metal concentrations in worker honeybees (µg g^{-1} dm) in samples from three locations. Concentration values are calculated as sample means over the entire study period (7 July - September 2006).

Element	Maastricht	Buggenum	Hoek van Holland
Al	9.17[a]	9.33[a]	9.07[a]
As	0.72[a]	0.73[a]	0.69[a]
Cd	0.16[a]	0.21[a]	0.11[a]
Co	0.11[a]	0.21[b]	0.10[a]
Cr	0.21[a]	0.22[a]	0.22[a]
Cu	17.66[a]	13.75[a]	14.22[a]
Li	0.02[a]	0.02[a]	0.02[a]
Mn	35.08[a]	37.97[a]	30.99[a]
Mo	0.79[a]	0.53[a]	0.54[a]
Ni	0.30[a]	0.31[a]	0.31[a]
Pb	0.45[a]	0.42[a]	0.55[a]
Sb	0.12[a]	0.11[a]	0.11[a]
Se	1.36[a]	1.29[a]	1.19[a]
Sn	0.50[a]	0.51[a]	0.51[a]
Sr	1.42[ab]	0.90[a]	1.61[b]
Ti	0.33[a]	0.28[a]	0.34[a]
V	0.03[a]	0.02[a]	0.15[b]
Zn	74.72[a]	79.59[a]	66.70[a]

2.3.2 Comparison with previously reported concentrations

For Al, Co, Li, Mo, Sb, Sn, Sr, Ti and V no previous reports on their concentrations in adult honeybees have been published. The ranges of the concentrations of these metals as found in our study are as follows: Al, 4.6 – 15.52 μg g^{-1}; Co, 0.08 – 0.33 μg g^{-1}; Li, 0.01 – 0.05 μg g^{-1}; Mo, 0.36 – 1.16 μg g^{-1}; Sb, 0.07 – 0.19 μg g^{-1}; Sn, 0.44 – 0.76 μg g^{-1}; Sr, 0.70 – 2.18 μg g^{-1}; Ti, 0.09 – 0.55 μg g^{-1}; and V, 0.006 – 0.31 μg g^{-1}. For As, Cd, Cr, Cu, Mn, Ni, Pb, Se and Zn published reports on concentrations in adult honeybees are available from a wide variety of sampling locations. These values are presented in Table 3, together with the ranges of concentrations found in our study. The method of analysis to determine metal concentrations in bees was either ICP-AES, as in our study, or Atomic Absorption Spectrometry (AAS). Kump et al. (1996) compared both methods for Cr, Cu, Mn, Ni, Pb and Zn and found only significant differences between these methods for Cr. The concentrations of the metals measured in our study are all within the bandwidth of the values reported in the literature, with the exception of Mn and Se. The concentrations we found for these two metals are lower than reported from other studies, but in the same order of magnitude. In general, the metal concentrations in the present study were at the lower or middle-lower end of the spectrum of concentration values found in other studies and often match the range of concentrations reported for supposedly relatively 'clean' locations. This indicates that the level of metal pollution at our three study locations was relatively low. The, in absolute terms, high concentrations of Cu, Mn and Zn are comparable to values found in other studies and are most likely due the relatively high natural concentrations of these metals in pollen (Lambers et al., 1998), on which the bees feed.

Table 3. Metal concentrations in adult honeybees in the current and previous studies.

Element	Current study	Previous studies		
	Concentration range (µg g^{-1})	Concentration range (µg g^{-1})	Comments	Reference
As	0.67 – 0.83	<0.5 – 12.5	72 sites (rural – urban)	Bromenshenk et al. 1985
		< 0.1	hives without CCA	Kalnins and Detroy (1984)
		0.77 – 1.11	hives with CCA	Kalnins and Detroy (1984)
Cd	0.05 – 0.75	<0.6 – >1.8	72 sites (rural – urban)	Bromenshenk et al. 1985
		2.89 – 3.43	non-contaminated sites	Conti & Botré (2001)
		2.87 – 4.23	sites in city centre/near highway	Conti & Botré (2001)
		0.03 – 0.18	control sites	Fakhimzadeh & Lodenius (2000)
		0.05 – 1.2	industrial sites	Fakhimzadeh & Lodenius (2000)
		1.1 – 1.9*	near crossroad with heavy traffic	Leita et al. (1996)
		0.14 – 0.16*	agricultural-forest region	Roman (2005)
		0.10 – 0.17*	industrialized region	Roman (2005)
		0.16 – 1.34	relatively clean locality	Veleminsky et al. (1990)
		0.74 – 1.75	industrial locality	Veleminsky et al. (1990)
Cr	0.15 – 0.28	0.054 – 0.080	non-contaminated sites	Conti & Botré (2001)
		0.052 – 0.116	sites in city centre/near highway	Conti & Botré (2001)
		1.4 ± 0.2*	different locations	Kump et al. (1996)
		< 0.06 – 0.34	hives without CCA	Kalnins and Detroy (1984)
		0.58 – 0.8	hives with CCA	Kalnins and Detroy (1984)

Element	Current study	Previous studies		
	Concentration range (μg g^{-1})	Concentration range (μg g^{-1})	Comments	Reference
		<0.1 – 3.6	national park	Porrini et al. (2002)
		<0.1 – 1.2	city centre	Porrini et al. (2002)
		0.05 – 0.18*	agricultural-forest region	Roman (2005)
		0.16 – 0.23*	industrialized region	Roman (2005)
Cu	11.65 - 19.77	13 – 15	control sites	Fakhimzadeh & Lodenius (2000)
		14 – 27	industrial sites	Fakhimzadeh & Lodenius (2000)
		35.7 ± 1.5*	different locations	Kump et al. (1996)
		8.68 – 9.70	hives without CCA	Kalnins and Detroy (1984)
		9.86 – 10.5	hives with CCA	Kalnins and Detroy (1984)
		15.16 – 30.55	relatively clean locality	Veleminsky et al. (1990)
		31.89 – 37.68	industrial locality	Veleminsky et al. (1990)
Mn	20.69 – 50.80	75.7 ± 5.6*	different locations	Kump et al. (1996)
Ni	0.19 – 0.47	0.12 – 0.42	national park	Porrini et al. (2002)
		0.13 – 0.43	city centre	Porrini et al. (2002)
		0.27 – 0.42*	agricultural-forest region	Roman (2005)
		0.36 – 0.50*	industrialized region	Roman (2005)
Pb	0.19 – 1.67	0.52 – 1.00	non-contaminated sites	Conti & Botré (2001)
		0.64 – 1.25	sites in city centre/near highway	Conti & Botré (2001)
		0.58 – 0.62	control sites	Fakhimzadeh & Lodenius (2000)

Element	Current study	Previous studies		
	Concentration range (µg g^{-1})	Concentration range (µg g^{-1})	Comments	Reference
		0.27 – 1.4	industrial sites	Fakhimzadeh & Lodenius (2000)
		1.4 – 3.0*	near crossroad with heavy traffic	Leita et al. (1996)
		0.15 – 0.55	national park	Porrini et al. (2002)
		0.45 – 0.95	city centre	Porrini et al. (2002)
		1.5 – 30	far from – near busy highway	Pratt & Sikorski (1982)
		0.28 – 0.29*	agricultural-forest region	Roman (2005)
		0.64 – 1.01*	industrialized region	Roman (2005)
		0.58 – 2.47	relatively clean locality	Veleminsky et al. (1990)
		3.68 – 9.28	industrial locality	Veleminsky et al. (1990)
Se	1.15 – 1.53	1.84 – 2.38*	agricultural-forest region	Roman (2005)
		2.16 – 5.98*	industrialized region	Roman (2005)
Zn	61.14 – 100.64	55 – 73	control sites	Fakhimzadeh & Lodenius (2000)
		59 – 100	industrial sites	Fakhimzadeh & Lodenius (2000)
		202 ± 5*	different locations	Kump et al. (1996)
		52.5 – 76.2*	near crossroad with heavy traffic	Leita et al. (1996)
		90.34 – 188.72	relatively clean locality	Veleminsky et al. (1990)
		153.34 – 204.4	industrial locality	Veleminsky et al. (1990)

** ICP-AES analyses*

2.3.3 Sources of metals in the hive environment

The commercial-type beehives used in our experiment have metal or metal-based components, such as stainless steel frame holders and wood preserving coatings. ICP-AES analysis of samples from the frame holders and from paint of the landing board at the hive entrance, revealed traces of As, Cr, Cu and Ni in both types of material (L. Goessen, pers. comm.). Thus, we cannot exclude that at least part of the load of these metals in the sampled bees originated from hive-associated sources. Kalnins and Detroy (1984) studied the effect of the use of the wood preservative CCA (chromate copper arsenate) in hives on the concentrations of As, Cr and Cu in bees. They found that the use of CCA significantly enhanced the concentrations of As and Cr in bees, but the concentrations of Cu were not significantly affected (Table 3). The lack of effect on Cu is probably due to the much greater importance of pollen as a source of this metal in bees. The concentrations of As found in our study correspond with those from hives treated with CCA in Kalnins and Detroy's study (1984) and are therefore probably the consequence of exposure to hive-associated sources rather than to sources in the external environment. As for Cr, the concentrations found in our study correspond with those from hives not treated with CCA, suggesting that the Cr-containing materials of the hive were not an important source of contamination in this case.

2.4 Conclusion

Our results indicate that honeybees can serve to detect temporal and spatial patterns in environmental metal concentrations, even at relatively low levels of pollution. A restriction on the potential use of apiculture for bio-monitoring of metal pollution is posed by the application of metal components and metal-based wood preservatives in commercial-type beehives.

A next step will be to determine sources of variation in metal concentrations. For that purpose, larger scale studies are required that allow for detailed statistical analysis. For example, we will conduct a follow-up study on spatial variation of metal concentrations in honeybees covering 150 locations across the Netherlands, which will be analyzed with geostatistical methods.

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Chapter 3

Assessment of the potential of honeybees (*Apis mellifera* L) in biomonitoring of air pollution by cadmium, lead and vanadium

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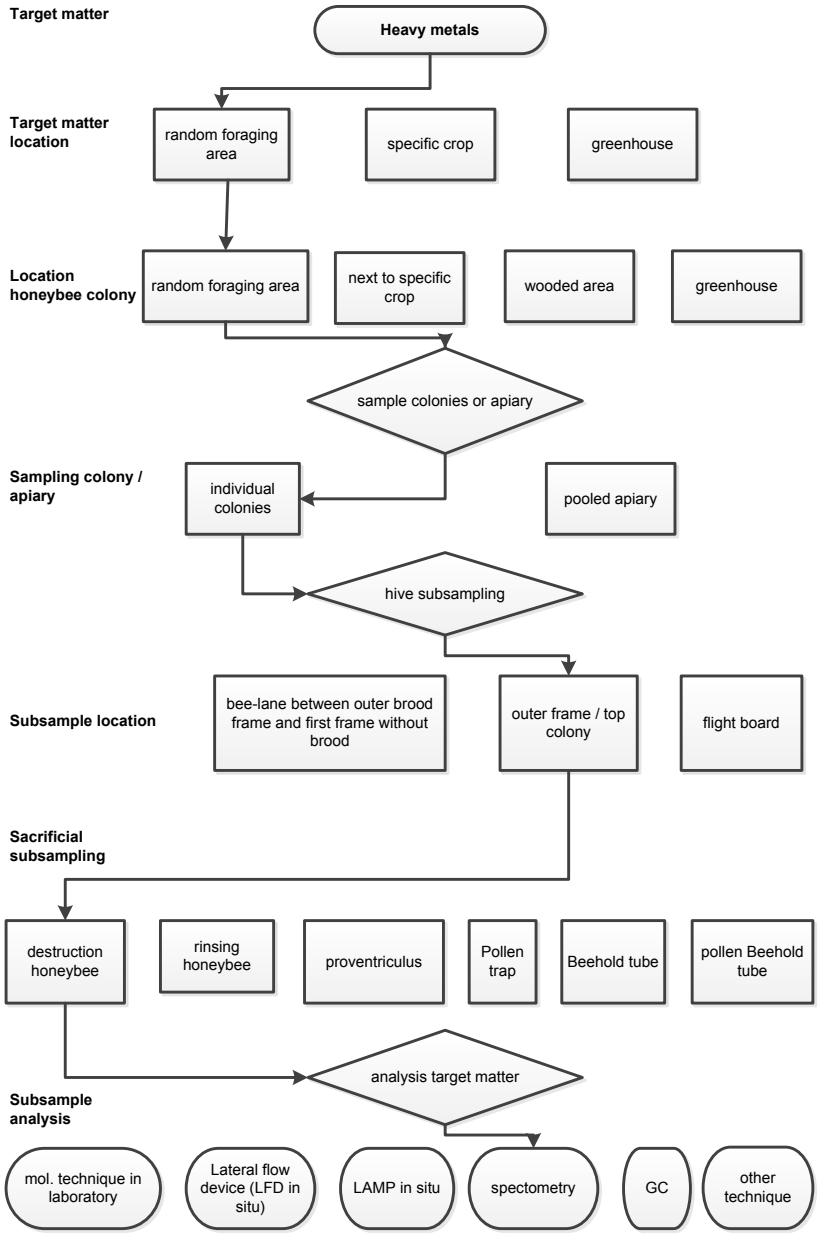


Figure I. Bio-indication flow chart: Assessment of the potential of honeybees (*Apis mellifera* L) in biomonitoring of air pollution by cadmium, lead and vanadium

Abstract
The aim of our study was to explore whether honeybees (*Apis mellifera* L) could be used as a reliable alternative to the standard mechanical devices for monitoring of air quality, in particular with respect to the concentration of the heavy metals cadmium (Cd), lead (Pb) and vanadium (V). We therefore tested whether the concentrations of these metals in adult honeybees and in ambient air were positively correlated, and whether differences in concentration between locations were similar for bees and air. On the basis of our measurements, conducted over a two-month period at three distinct locations in the Netherlands with each three replicate honeybee colonies placed next to mechanical monitoring devices, we conclude that a significant positive relationship between the concentrations in bees and in air could only be established for V. Also, only in case of V, the differences between the three locations in mean concentration were similar for bees and air. Both outcomes are probably due to the relatively large range over which the concentrations of V varied, both in bees and in air, as compared to Cd and Pb. However, for V, as well as for Cd and Pb, the concentrations in ambient air were about two orders of magnitude below the established air quality standards. We therefore conclude that in the Netherlands, both variation and levels of the atmospheric concentrations of these metals are too low to establish a relationship between the concentration in bees and in air that is useful to present honeybees as an alternative to mechanical devices in monitoring of air pollution. However, in countries with larger variation and higher levels of the atmospheric concentrations of these metals further exploration of the potential of honeybees in biomonitoring of air pollution may be worthwhile.

3.1 Introduction

Honeybees (*Apis mellifera* L) are potentially highly useful to monitor environmental pollution, given their worldwide usage for honey production and pollination and their wide-ranging foraging behavior (Bromenshenk & Preston, 1986; Raeymaekers, 2006). Not surprisingly, studies on the use of honeybees and bee products for environmental monitoring have a relatively long history, dating back to at least 1935 (Crane, 1984). Environmental pollutants included in these studies were, among others, heavy metals, which the bees may take up from all environmental compartments: vegetation, soil, air and water (Bromenshenk et al., 1985; Conti & Botré, 2001; Leita et al., 1996; Mihaly et al., 2012; Porrini et al., 2013). A recent study by van der Steen et al., 2012[a]) indicated that adult honeybees can serve to detect temporal and spatial patterns in environmental concentrations of a wide range of heavy metals. For seven of the metals included in their study, viz., arsenic (As), cadmium (Cd), chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb) and vanadium (V), air quality standards have been established by national, European and international authorities (EU, 2008; Staatsblad, 1997; WHO, 2000). The standards concern Maximum Tolerated Risk (MTR) values for the concentrations of these metals in the air, and aim to prevent adverse effects on human health from air pollution. In the Netherlands, a country-wide air quality monitoring system is in place, using fixed-point mechanical monitoring devices to assess whether air quality standards are met (De Jong & Janssen, 2010).

The aim of the study we report here was to explore whether adult honeybees could be used as a reliable alternative to the standard mechanical monitoring devices to assess significant changes in the concentration of heavy metals in the air and to detect possible exceeding of MTR values. To that end, we designed an experiment in which bee colonies were placed side-by-side with mechanical monitoring devices at various locations over a prolonged period, during which regular samples were taken from bees and air and analyzed for heavy metals. We considered that bees would be a reliable alternative in case the metal concentrations found in bees and in air would positively correlate, and when possible significant differences in concentration between locations would be similar for bees and air. In contrast to Balestra et al. (1992) who studied honey, pollen and bee larvae, we chose to study the metal concentrations in adult worker bees as these are considered to provide more sensitive, reliable and up-to-date information about exposure of bees to metals in the environment than metal concentrations in pollen, honey and larvae (Bromenshenk et al, 1985; Fakhimzadeh & Lodenius, 2000; Jones, 1987; Mihaly Cozmuta et al., 2012; Porrini et al., 2003[b];

Veleminsky et al., 1990). We report here the results for the heavy metals Cd, Pb and V. The heavy metals As, Cr, Mn, and Ni are not included, because these metals either occur in relatively high natural concentrations in bee food sources such as pollen (Lambers & Chapin, 1998), or occur in commonly used components of beehives (Steen et al., 2012[a]).

3.2 Materials and methods

The bio-indication scheme is presented in the flow chart on page 62 (Figure I).
At three distinct locations in the Netherlands, honeybee colonies were placed next to standard air quality monitoring devices. Each honeybee colony was sampled four times over a two-month period. The concentrations of metals (Cd, Pb and V) in the sampled honeybees were compared with the concentrations of these metals in the air as determined with the standard monitoring devices. Per metal, statistical analyses were conducted to assess the relationship between the concentrations in honeybees and in air.

3.2.1 Study locations

Location Maastricht, Limburglaan. Maastricht is an urban area with cement industry and glass industry, and is located close to large industrial areas such as Liège in Belgium. The honeybee colonies were placed near the city centre, on the roof of the provincial environmental research laboratory.
Location Buggenum, Dorpstraat. Buggenum is a village in a rural area about 60 kilometres north of Maastricht. In Buggenum bricks are produced and a large electric power plant is situated next to the village. This plant is powered by coal, natural gas and biomass.
Location Hoek van Holland, Prins Hendrikstraat. Hoek van Holland is situated in the Rijnmond region at the river mouth of the Nieuwe Waterweg, at the North Sea coast. The Rijnmond region includes the port of Rotterdam and a large industrial area where, among others, petrochemical industry, tank storage and tank transfer, and waste treatment plants are situated.

3.2.2 Honeybee sampling method

Honeybee (*Apis mellifera* L) colonies were kept from winter until summer in the same apiary in Wageningen (The Netherlands) until distribution over the three locations. Three honeybee colonies (replicates) were placed at each location. The colonies were kept in one-storey wooden hives with ten frames (Simplex measures NEN 061-50). This is the most common, commercially used type of hive in the Netherlands. Each honeybee colony was sampled four times with 14-day intervals during a two-month period

(7 July – 1 September 2006). A random sample of 100 to 150 worker honeybees was taken from the first frame next to the brood nest that was occupied with bees but without brood. Hive bees were sampled rather than forager bees from the flight entrance as this is much more practical and forager bees constitute a stable fraction of the bees on the sampled frames (Steen et al., 2012[b]). Bees sampling was done by brushing bees with a plastic brush into a plastic container. The samples were transported in a cooler box and stored in the freezer at −20±5 ºC until analysis.

3.2.3 Measurement of metal concentrations in bees

From each sample (i.e., from each combination of colony, sampling date and location), 25 frozen worker bees were taken at random. The bees were subsequently weighed, dried during 24 hours at 120 ºC, weighed, and destructed by boiling the sample at 170 ºC in a mixture of 25 ml HNO_3 (70%) and HCl (37%) at a ratio of 1:3 (aqua regia). The resulting liquid was topped up to 50 ml with demi-water. The chemical analyses on metals were carried out by the environmental research laboratory of the Province of Limburg (The Netherlands), using the inductive coupled plasma – atomic emission spectrometry (ICP-AES) technique. Five milliliters of the 50 ml was filtered over a cotton wool filter and analyzed using ICP-AES. The measured metal concentrations ($ng.mL^{-1}$) were converted to $ng.g^{-1}$ dm bee (ppb) with a conversion factor (volume sample / (weight bees × mean percentage dry weight)), resulting in ppb metal in dry weight which was subsequently converted to micrograms per gram dry material bee ($\mu g.g^{-1}$ dm). The overall weight loss of the bee samples as a result of the drying process was 68%.

3.2.4 Measurement of metal concentrations in air

At Maastricht and Buggenum, measurements of the atmospheric concentration of the metals were taken daily with fixed mechanical monitoring devices operated by the provincial environmental and water research service (Hoofdgroep Milieu en Water, Bureau Onderzoek en Advies, Provincie Limburg). The devices are high volume air samplers, which press 680 m^3 air through glass-fibre filters in 24 hours. At Hoek van Holland, the measurements were taken at six-day intervals with a fixed monitoring device operated by the regional environmental management service (Dienst Centraal Milieubeheer Rijnmond, DCMR). Here, the device is a high volume air sampler, which presses 668 m^3 air through quartz filters in 24 hours. The filters were analysed for the metals in the laboratory using the same technique as for the bee samples (ICP-AES). The concentrations of heavy metals in the air were expressed in nanograms per m^{-3} ($ng.m^{-3}$) air.

3.2.5 Statistical analyses

The aim of the statistical analyses was to assess for each metal the relationship between the concentrations in honeybees and in air. The analyses were performed in Genstat 16^{ed}. In a first analysis, the relationship between the concentration of a metal in honeybees ($\mu g.g^{-1}$) and the concentration of the same metal in the air ($ng.m^{-3}$) was assessed with logarithmic regression. For the analysis, the metal concentration in bees was calculated as the average value of the three bee colonies (replicates) per location and sampling date, and the metal concentration in the air was calculated as the average of all air samples taken during the 14-day period preceding the sampling of the bees. All averages of concentration data were calculated by log-transformation of the raw data followed by back-transformation of the average of the log-transformed data. The regression analysis was conducted per metal, with concentration in bees as the dependent factor and concentration in air as the independent factor. The data of the four sampling times and three locations were pooled on the assumption that the relationship between the metal concentration in bees and air should be independent of time and place to be useful in biomonitoring. The data were checked for significant outliers with Grubbs' test (alpha=0.05) and analyses were conducted with and without outliers. As outliers did not affect the conclusions, we did not exclude outliers from the data and analyses. A second analysis concerned the correspondence in spatial variation between the metal concentrations in honeybees and air. Per metal, a Generalized Linear Mixed Model (GLMM) analysis was done assuming a lognormal distribution of the concentration data. Spatial differences in concentration were assessed separately for honeybees and air, by comparing, between locations, the mean values of all measurements during the entire two-month study period. Differences in concentrations between locations were considered significant at P values ≤ 0.05, using Tukey's Multiple Comparisons test.

3.3 Results and discussion

Regression analysis of pooled data (covering four sampling times and three locations) yielded a highly significant, positive relationship between the concentration of V in honeybees and in air. Another highly significant, but negative relationship was found between the concentration of Cd in honeybees and in air. No significant relationship was found between the concentration of Pb in honeybees and in air. For the significant relationships, the regression lines are included in figure II and the corresponding regression equations and other statistics are presented in Table 1.

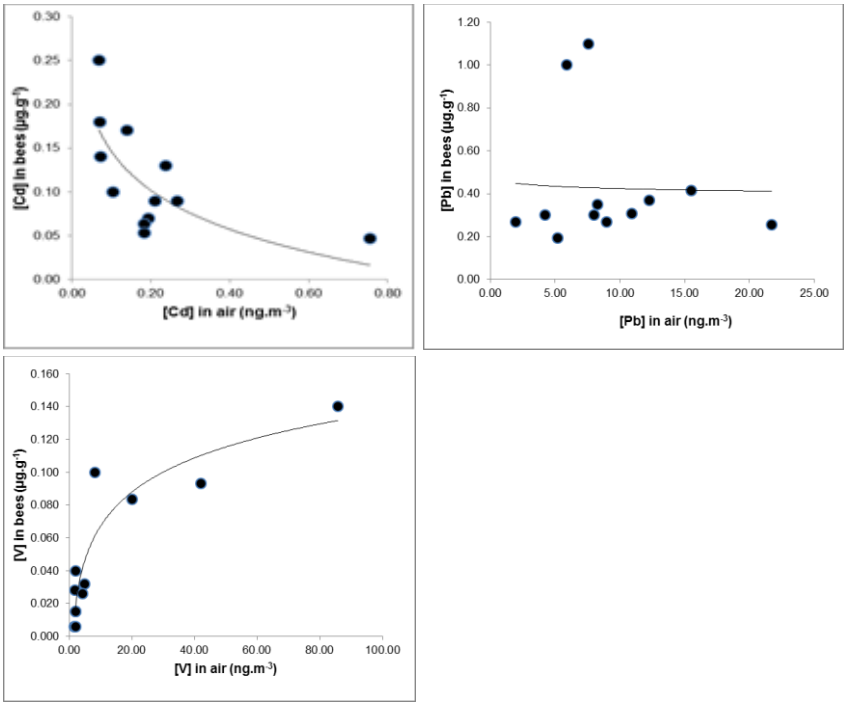


Figure II. Scatter plots (with regression lines) of concentrations of V, Cd and Pb in air ($ng.m^{-1}$) versus V, Cd and Pb ($\mu g.g.bee^{-1}$ dm). Data points represent the pooled results from all sampling dates and locations.

Table 1. Logarithmic regression of concentrations of Cd, Pb and V in honeybees (y, $\mu g.g^{-1}$ dm) and in air (x, ng.m-3): regression equations, P values of regression and the R^2 values (percentage variance accounted for)

Element	Regression equation	P	R^2
Cd	y = -0.06ln(x)-0.001	0.008	48%
Pb	y = -0.016.Ln(x) + 0.46	0.92	0%
V	y = 0.030ln(x) -0.0017	< 0.001	84%

The correspondence in spatial variation between the metal concentrations in honeybees and air was tested. Table 2 presents the results of the analyses of spatial variation in the metal concentrations, for honeybees and air. In honeybees, significant differences in concentration between locations were found for Cd as well as for V. In air, the mean concentrations over the entire two-month study period of Cd, Pb and V, differed significantly between the three locations. Only in case of V, there was correspondence in spatial variation between the metal concentrations

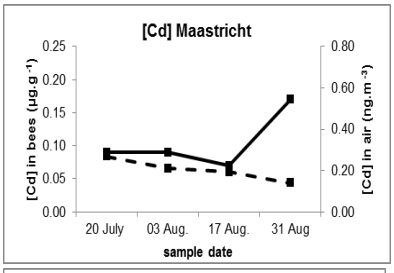
in honeybees and air, with significantly higher concentrations in Hoek van Holland than in Maastricht and Buggenum. In case of Cd, there was no correspondence in spatial variation between the metal concentrations in honeybees and air. For honeybees, the highest Cd concentrations were found in Buggenum, whereas for air, the lowest Cd concentrations were found there.

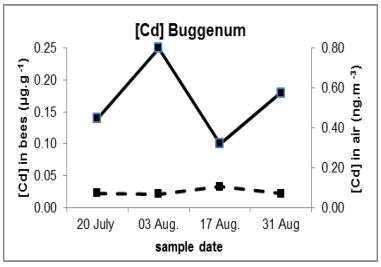
Table 2. Metal concentrations in worker bees (µ.g $^{-1}$ dm) and air (ng.m $^{-3}$) from three locations, calculated as sample means over the entire sampling period (7 July – 1 September 2006).

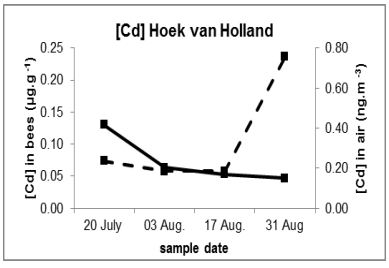
Element	Maastricht	Buggenum	Hoek van Holland
[bees]			
Cd	0.10 ^{ab}	0.16 ^{b}	0.06 ^{a}
Pb	0.33 ^{a}	0.36 ^{a}	0.41 ^{a}
V	0.02 ^{a}	0.013 ^{a}	0.10 ^{b}
[air]			
Cd	0.17 ^{b}	0.07 ^{a}	0.30 ^{b}
Pb	12.53 ^{b}	7.55 ^{a}	4.89 ^{a}
V	1.81 ^{a}	2.18 ^{a}	27.94 ^{b}

Different superscript letters indicate per location (row) statistically significant differences between sample means.

The concentrations of Cd, Pb and V measured in honeybees and air over the test period are presented in figure III separately for the three study locations.







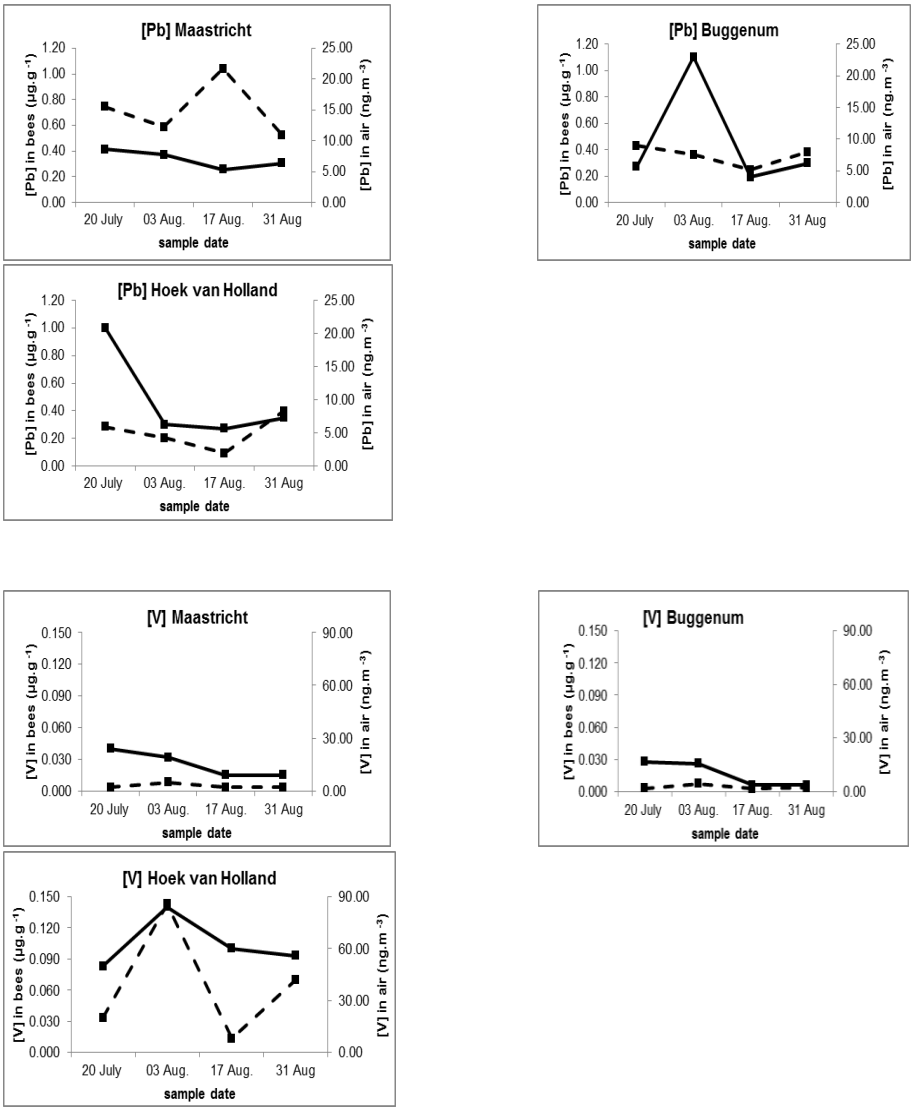


Figure III. Concentrations of Cd, Pb and V in worker honeybees (µg.g $^{-1}$ dm, drawn line) and air (ng.m $^{-3}$) , dashed line) in samples from three locations. The sample date (x axis) refers to the sampling period (air) and sample dates (honeybees). The sample dates of the honeybees correspond with the sampling periods of air (between brackets), as follows: 20 July (7-20 July); 03 Aug (21 July–3 August); 17 Aug (4–17 August); 31 Aug (18–31 August).

On the basis of our measurements, conducted at three distinct locations in the Netherlands over a two-month period, a significant positive relationship between the concentrations in bees and in air could only be established for vanadium (Table 2). Also, only in case of vanadium, the differences between the three locations in mean concentration were similar for bees and air (Table 2). Both outcomes are probably due to the relatively large range over which the concentrations of V varied, both in bees and in air, as compared to Cd and Pb. For V, measured concentrations differed by 1-2 orders of magnitude, whereas for Cd and Pb this was less than one order of magnitude (Figure I, Table 1). The larger the variation in measured concentrations, the higher the probability to detect significant, meaningful relationships.

With regard to the potential of honeybees in biomonitoring of air pollution by heavy metals the results for V are promising. However, the concentrations of V in air measured in our study were about two orders of magnitude below the air quality standard for V (1000 ng m^{-3}) (De Jong & Janssen, 2010), and extrapolation of a statistical relationship beyond the range over which it is established is not allowed. To warrant a conclusion on the reliability of honeybee biomonitoring as an alternative to the standard mechanical monitoring, the positive relationship found between the concentrations of V in bees and in air, should thus also be tested for situations in which the concentration of V in air exceeds 1000 ng.m^{-3}.

For Cd and Pb, our results seem to indicate that there is no potential for honeybees in biomonitoring of air pollution by these heavy metals. As indicated above, however, this may be due to the limited range over which the concentrations of Cd and Pb varied in our study. The work of Bromenshenk et al. (1988) points in this direction. They found that the concentrations of Cd and Pb in weekly sampled worker bees were significantly and positively correlated with the concentrations in air during the same period. Unfortunately, Bromenshenk et al. (1988) did not present measurement data or any other quantitative results, but, as they located the bee colonies and air sampler near a lead smelting complex, the concentrations of Cd and Pb in the air were presumably high. In our study, the concentrations of Cd and Pb in air were relatively low and, as for V, about two orders of magnitude below the air quality standards (5 ng m^{-3} for Cd and 500 ng m^{-3} for Pb, (De Jong & Janssen, 2010). Thus, also for Cd and Pb, further testing of the relationship between the concentrations in bees and in air should be done under conditions with more variation and concentration levels exceeding the MTR values.

3.4 Conclusion

Our results indicate that in the Netherlands, both variation and levels of the atmospheric concentrations of these metals are too low to establish a useful relationship between the concentration in bees and in air. Under these conditions, bees cannot be used as a reliable alternative to the current system in place to meet legal monitoring requirements. However, our results positively indicate for V, and do not exclude for Cd and Pb, that a relationship between the concentrations in bees and in air can be established and applied for biomonitoring purposes. This should be done under conditions of relatively high variation, with atmospheric concentrations of these metals differing by several orders of magnitude, and maximum levels exceeding the MTR values. In countries where these conditions apply, we consider further exploration of the potential of honeybees as an alternative to the establishment of a costly mechanical monitoring network worthwhile.

Acknowledgements
We thank Dr M. Severijnen, Dr J. Pijnenburg (Provincie Limburg) and Dr P. Kummu (DCMR Rijnmond) for their help in selecting the study locations, Ing. L. Goessen (Province of Limburg) for the chemical analyses of the samples, Mr J. Withagen (Wageningen UR) for the statistical analyses.

Chapter 4

"Think regionally, act locally" Metals in honeybee workers in the Netherlands

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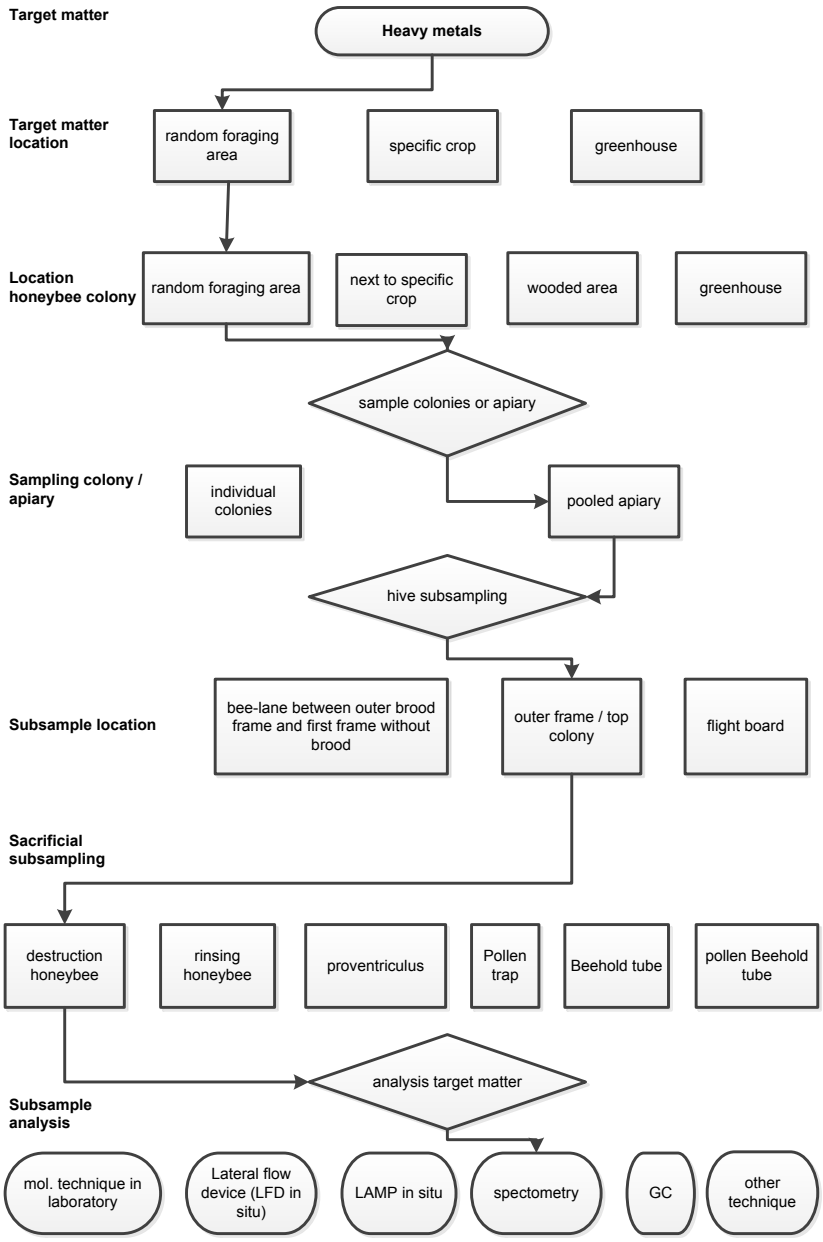

Figure I. Bio-indication flow chart: Think regionally, act locally. Metals in honeybee workers in the Netherlands

Abstract
In June 2008 a surveillance study for metals in honeybees was performed in the Netherlands. Randomly 150 apiaries were selected. In each apiary five colonies were sampled. Per apiary the hive samples were pooled. The apiary sample was analysed for Al, As, Ba, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Sb, Se, Sn, Sr, Ti, V and Zn. All metals could be detected in all apiaries. As, Li, Sb, Sn and V were detected in part of the apiaries. The overall picture showed a regional pattern. In apiaries in the East of the Netherlands, Al, Ba, Cr, Mn, Mo, Ni, Se and Ti are found in higher concentrations compared to the West. In-region variation was demonstrated, indicating local effects. The vicinity of the apiaries was mapped afterwards and characterised as land uses > 50% agricultural area, > 50% wooded area, > 50% urban area and mixed land use within a circle of 28 km^2 around the apiary. The results indicated that in apiaries located in >50% wooded areas, significant higher concentrations of Al, Ba, Cd, Cr, Cu, Li, Mn, Mo, Ni, Sb, Sr, Ti and Zn were found compared to agricultural- urban and mixed land use areas.
We conclude that 1) the ratio between metal concentrations varies per region demonstrating spatial differences; 2) there is in-region local variation per metal. The results indicate the impact of land use on metal concentrations in honeybees. For qualitative bio-indication studies, regional- local-and land use effects should be taken into account.

4.1 Introduction

Along with collecting nectar, pollen, water and propolis, honeybees pick up particles deposited in the flowers and other places where bees collect resins (propolis) and water. Honeybees also collect the sweet aphid secretion called honeydew, from the leaves. Additionally, to pollen collected from the anthers, the branched hairs on the bee's body easily hold non-floral particles originating from atmospheric deposition. In this way each honeybee can act as an environment micro-sampler and a honeybee colony as a sampler unit. In the active foraging period of the honeybee colony, about a quarter of the colony's population is a forager bee. The number of foragers, actively collecting food, depends on the colony size, the colony's need for pollen, nectar, water and propolis, the availability of food and the time of year. The number of foraging trips varies from some hundreds to many thousands of trips per day resulting in hundreds to many thousands micro-samples accumulated in the honeybee colony in the hive. The feature of the honeybee of collecting unintentionally non-floral particles makes the honeybee suitable for qualitative bio-indication, providing information about the environment.
Metals are a natural component of the bee's food. In "Honey, a comprehensive survey" by Crane (1979) an overview is presented of metals in honey, showing that dark honeys, often partly made from honeydew, contain higher concentrations of metals compared to light ones. For example, the average Mg in light honey is 19 ppm and in dark honeys 35 ppm. For Cu the averages are 0.29 and 0.56 respectively. The concentrations range significantly; the lowest Fe concentration presented is 0.70 ppm and the highest is 33.50 ppm, both in dark honey. In a honey study in south east Anatolia the mean concentrations of Mg, Cu, Mn, Zn and Co were 33, 1.8, 1.6, 2.7 and 1.0 ppm (Yilmaz & Yavuz, 1999). Latorre et al. (1999) classified honeys based on the metal content data. Mean concentrations metals in pollen of 20 samples were, determined in a study in Spain by Serra Bonheve & Escola Jorda (1997) Fe, 39.2 ppm; Zn 33.9 ppm; Cu 8.7 ppm Mg 432.2 ppm. Campos et al, 2008 present the range of metals in dried pollen for K: 4000 – 20000 ppm; Mg: 200 – 3000 ppm; Ca: 200 – 3000 ppm, P: 800 – 6000 ppm; Fe: 11 – 170 ppm; Zn: 30 – 250 ppm; Cu: 2 - 16 ppm Mn: 20 – 110 ppm in the study of detailed composition of bee collected pollen. The sources of metal detected in honeybees are nectar, honeydew, pollen plus possible atmospheric deposition of metal containing particles. Part of the metals will be in the bee because of ingestion of food and part on the exterior of the bee as pollen and non-floral particles. Analysing the complete bee the result is the sum of what is in and on the bee. In the current study this is referred to as metals in the bee. It is obvious that any analysis of bees on heavy

metals results in detecting metals in varying concentration ranges. Therefore, in bio-indication studies only significant exceeded concentrations of metals in honeybees studied under defined site conditions, indicate an extra exposure of bees to heavy metals and may draw attention for further studies. Concentrations of heavy metals show significant temporal and spatial variation (Lambert et al., 2012[b]; Perugini et al., 2011; Ruschioni et al., 2013; Satta et al., 2012; Steen et al., 2012[a]). Bio-indication studies revealed that high heavy metal concentrations can only be detected in live honeybees and not in honey and dead bees (Ruschioni et al., 2013). The mechanism behind the difference in metal concentrations in dead and live bees was not part of this study. The life bees were forager bees and the dead ones died in the hive; this may explain the difference in exposure to heavy metals brought in by the foragers. According to Satta et al. (2012) sampling foragers gives the best result to detect heavy metals in bees. Land use affects metals concentrations in the honeybee. Apiaries in urban and landscapes with hedgerows contained higher concentrations Pb in honeybees than the ones in cultivated and island landscapes (Lambert et al., 2012[b]). Weather conditions also affect the concentration of heavy metals in bees; dry weather results in higher concentrations (Lambert et al., 2012[b]; Satta et al., 2012).

Most heavy metal bio-indication studies with honeybees are focused on a limited number of metals and performed at defined sites e.g. near motorways, airports, industrial sites, agricultural areas and land-fill sites. The results are compared to control sites (mostly urban sites or natural reserve parks) to demonstrate differences. Perugini et al. (2011) showed elevated Pb concentrations near the Ciampino airport of Rome compared to three nature reserves and a moderately polluted urban area. Forager bees in a post mining area in Sardinia contain more Cd and Pb than the ones in the control sites 50 km from the post-mining area (Satta et al., 2012).

The current study presented is to our knowledge the first national surveillance study conducted on 18 metals. The objective of this study was to collect data of the spatial variation of metal concentrations in honeybee colonies in the Netherlands. Afterwards the land use in the vicinity of the apiaries was mapped to evaluate the impact of the land use (urban, rural, agricultural and mixed sites) on the concentration of metals in honeybees. As sampling was done in June 2008, only spatial differences were studied. The study included the metals Al, As, Ba, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Sb, Se, Sn, Sr, Ti, V, and Zn. Following the definition of heavy metals being metals having a periodic system element number exceeding Fe (element number 26), Al, Li, Mn, Ti and V do not meet this definition.

Nevertheless, these metals are included in this study and the term metals in this article represent all metals including the heavy metals.

4.2 Material and methods

The bio-indication scheme is presented in the flow chart on page 74 (Figure I).

4.2.1 Number of apiaries to be sampled

The surveillance was set up to detect both the incidence of honeybee diseases and concentrations of heavy metals in honeybees in the Netherlands. The number of apiaries sampled was based on the probability to detect honeybee diseases at low prevalence. The number of apiaries to be sampled in order to detect bee diseases is calculated with the binomial probability theory equation $N = \frac{\ln(1-D)}{\ln(1-P)}$. N = sample size, ln = natural logarithm, D = probability (power) of detection, P = minimal proportion of bees carrying the pathogen (Pirk et al., 2013). With a probability (power) of 0.95 and a minimal proportion of 2% of the apiaries having a bee disease at low prevalence in the Netherlands, 148 apiaries must be sampled to detect at least one infected apiary. In this study 150 apiaries were sampled.

4.2.2 Number of colonies / pooled bee samples

Based on the variance of metal concentrations recorded in three hives per apiary in the Steen et al., (2012[a]) study, minimally three colonies should be sampled for a reliable mean apiary recording. Sampling was done by trained beekeepers. Per apiary five colonies were sampled by collecting about 100 bees from the outer brood frame. As the objective of the study was to obtain the incidence of metal in honeybees per apiary and not the difference between metal concentrations in bees of different colonies in an apiary, the samples were pooled resulting in one apiary sample. The samples were stored during transportation in a cooling box and next stored till analysis at −20°C.

4.2.3 Chemical analysis

The chemical analyses were performed by the environmental laboratory of the Province Limburg (Hoofdgroep Milieu and Water Bureau onderzoek en advies). Per pooled apiary sample 25 bees were picked randomly, weighed, dried for 24 h at 120 °C and destructed by boiling at 170 °C in *aqua regia*. The resulting liquid was topped up to 50 ml with demi water. After an overnight rest, the clear top liquid was analysed using ICP-AES. The resulting signals (nanogram per millilitre) were converted to nanograms per gram bee with a conversion factor (volume sample /

(weight bees x mean percentage dry weight)). This resulted in concentrations expressed as ppb per dry weight. The ppb per dry weight was subsequently converted to µg metal per gram dry matter bee.

4.2.4 Land use

Applying GIS÷ software÷ ArcGis 9.2, land use was mapped using LGN5-database (landgebruik. Unit postcode) in a 28 km^2 area around the apiary (radius approximately 3 km). The percentages of land use were calculated with the parameters: Code 7: arable land; Code 8: glass horticulture; Code 9: orchard; Code 11 wooded area; Code 16 water; Code 18 urban area; Code 25 infrastructure; Code 30 nature. All other land uses were combined as "mixed use". The foraging area circling the apiaries was then defined by the dominant land use type, combining the given land use definitions for categories: Agricultural, Wooded, Urban and rest/mixed land use. Areas covering ≥ 50% of one of these categories were classified as such.

The geographic distribution of apiaries sampled is presented in figure II.

Figure II. Location of apiaries sampled

4.2.5 Statistics

Of the metals, not detectable in an apiary sample, ½ Limit of Detection (LOD) value is set in the database. Per metal in the 150 apiary-dataset, the median, lower quartile (25%), upper quartile (75%), arithmetic mean, min/max, and standard deviation (sd) were calculated. The means and statistical differences of the metal concentrations per land use surrounding the apiary is calculated with ANOVA at a p-level 0.05. Differences between means, exceeding the Least Significant Difference (LSD) are considered significantly statistically different. This data set consisted of 147 apiaries

used for bee disease checks out of the 150 apiary-dataset. Of multiple apiaries owned by a one beekeeper one apiary was included in the disease and land use analysis. The calculations were done with Genstat 12 Ed.

4.2.6 Regions

The regions are based on the postal codes. In Table 1 per first postal code number and the corresponding part of the Netherlands is presented.

Table 1. General description of the Netherlands in postal code regions

postal code	general localisation of the regions
1	Northern part of Noord Holland and Gooi
2	Southern part of Noord Holland and northern part of Zuid Holland
3	Southern part of Zuid Holland and Utrecht
4	Zeeland and Western part of Noord Brabant
5	Mid and eastern part of Noord Brabant and the Northern part of Limburg
6	Mid and southern part of Limburg and region Nijmegen / Arnhem
7	Gelderland minus region Nijmegen / Arnhem, eastern part of Overijssel and
8	Western part of Overijssel and western part of Friesland
9	Eastern part of Friesland and Groningen

4.3 Results

4.3.1 Metals

The concentration of metals per gram dry matter worker bee of pooled samples per apiary is presented in table 2. The medium and mean differ slightly showing the data are not completely normally distributed; they are skewed to the higher concentrations. Nevertheless, the normal distribution appeared to be the best fitting distribution.

Table 2. Metals in honeybee workers (µg.g dm bee^{-1}) of pooled samples of five colonies per apiary of 150 apiaries

metal	median	lower quartile 25 percentile	upper quartile 75 percentile	mean	min / max	sd
Al*** (Aluminium)	15.55	11.88	22.90	17.75	4.95 / 43.90	8.01
As* (Arsene)	0.85	0.56	1.03	0.79	0.13** / 1.64	0.33
Ba (Barium)	1.84	1.30	2.40	2.05	0.27 / 8.68	1.25
Cd (Cadmium)	0.22	0.15	0.31	0.24	0.05 / 0.73	0.13
Co (Cobalt)	0.16	0.14	0.22	0.19	0.08 / 0.63	0.08
Cr (Chromium)	0.39	0.33	0.52	0.45	0.19 / 1.42	0.19
Cu (Copper)	19.25	17.2	22.5	20.00	11.70 / 32.2	4.13
Li* (Lithium)	0.01	0.01	0.03	0.03	0.01** / 0.13	0.02
Mn (Manganese)	154	81.70	226.00	167.70	31.30 / 524.00	106.40
Mo (Molybdenum)	0.68	0.55	0.84	0.75	0.35 / 5.28	0.44
Ni (Nickel)	0.55	0.41	0.76	0.60	0.13 / 1.48	0.26
Sb* (Antimony)	0.30	0.13	0.43	0.31	0.13** / 3.22	0.29
Se (Selenium)	1.96	1.46	2.56	2.10	0.77 / 4.37	0.81
Sn* (Tin)	0.35	0.27	0.44	0.39	0.13** / 3.30	0.34
Sr (Stontium)	1.80	1.33	2.15	1.82	0.66 / 4.59	0.69
Ti (Titanium)	0.42	0.30	0.58	0.48	0.10 / 2.99	0.32
V* (Vanadium)	0.03	0.013	0.05	0.04	0.01** / 0.32	0.04
Zn (Zinc)	95.75	83.50	114.00	100.4	56.60 / 170.00	22.65

* not detected in all apiaries. As, Li, Sb, Sn and V were not detected in respectively 7, 84, 62, 30 and 62 apiaries

** ½ LOD

*** Al was analysed in 149 samples, one analysis failed.

In figure II, the concentrations of the 18 metals and the 150 apiaries are presented as µg metal.dm bee^{-1} above and below the median (concentration minus median). In figure III, the median is set as 0. For reading the actual concentrations per apiary from figure II, the median (Table 2) should be added.

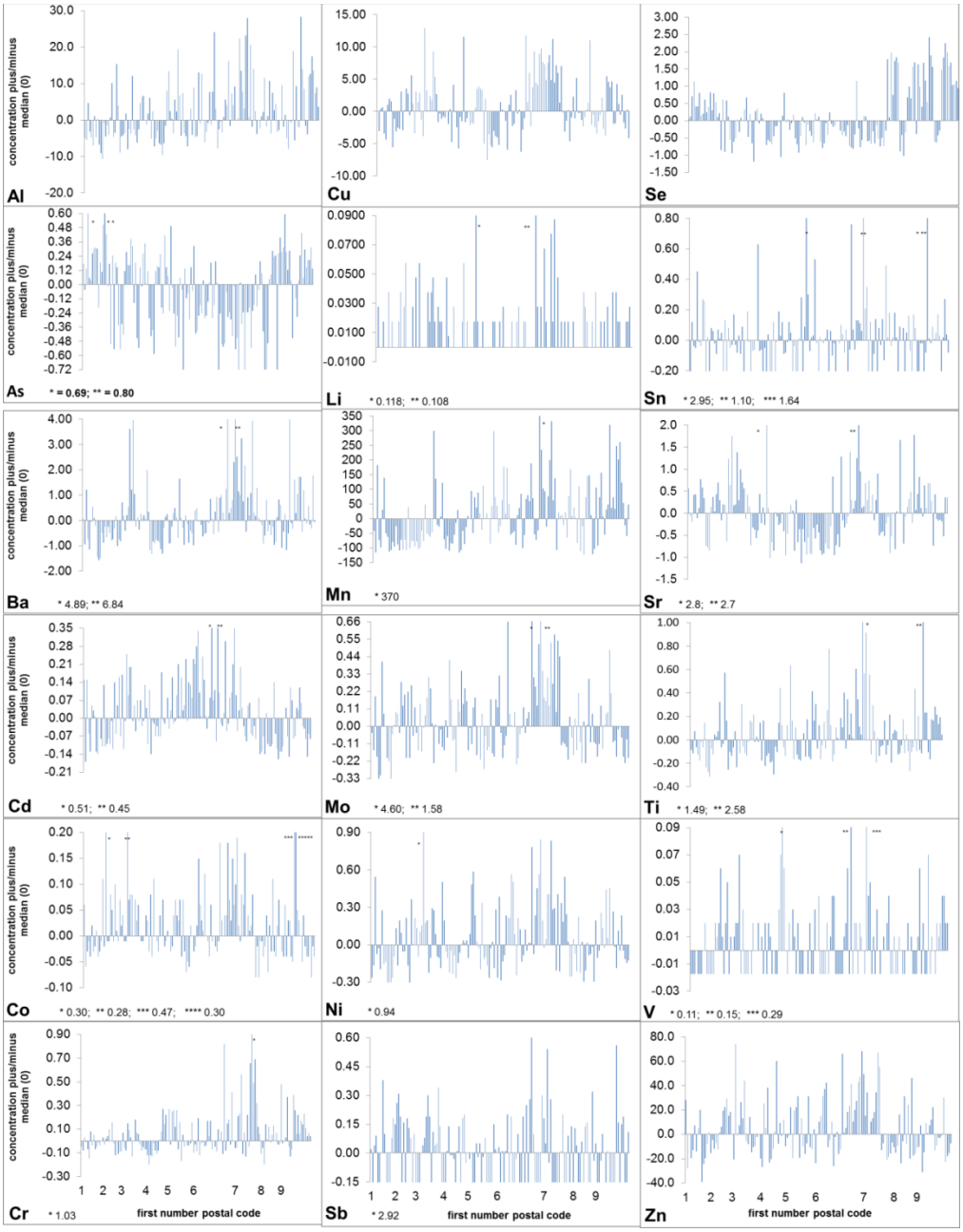

Figure III. The concentrations metals (µg. g dm bee^{-1}) displayed as concentrations above and below the median (Table 1). The median is set on 0. The results per region of the postal codes are shown between the subsequent numbers of the first number of the postal codes (Table 2). The exact even bars in Li (plus), Sb (minus), Sn (minus) and V (minus) show the analysis results are below the LOD and taken into the calculations as ½ LOD. The data exceeding the scale of the graphs are marked with an asterisk * and the values are displayed next to the metal symbols in the regarding graphs legend.

4.3.2 Land use

There is a significant difference in metal concentrations in bees in apiaries located at different land use sites. All metals except As, Se, Sn and V are recorded in significantly increased concentrations in > 50 % wooded areas. Metal concentrations in > 50 % agricultural areas, > 50 % urban area and mixed land use show no significant difference (< Least Significant Difference (LSD)). The mean metal concentrations per land-use are presented in Table 3.

Table 3. Metal in µg.g dm bee^{-1} per land use

Metal	> 50% agricultural area (n = 94)	> 50% woods (n = 7)	> 50% urban area (n = 16)	Mixed land use (n = 30)	LSD	P
Al	17.33	29.43	16.06	17.04	5.92	< 0.001
As	0.79	0.53	0.93	0.80	0.25	0.066
Ba	1.84	4.52	2.02	2.24	0.88	<.001
Cd	0.23	0.38	0.18	0.28	0.09	0.002
Co	0.18	0.26	0.19	0.18	0.06	0.041
Cr	0.45	0.66	0.38	0.42	0.14	0.007
Cu	19.58	26.40	20.01	19.46	3.03	<.001
Li	0.02	0.06	0.04	0.02	0.02	<.001
Mn	162.40	326.10	92.20	188.00	76.48	<.001
Mo	0.70	1.71	0.68	0.70	0.30	<.001
Ni	0.57	0.98	0.58	0.61	0.20	0.001
Sb	0.29	0.84	0.29	0.30	0.21	<.001
Se	2.18	1.77	2.02	2.05	0.63	0.518
Sn	0.40	0.47	0.40	0.37	0.26	0.908
Sr	1.75	2.6	1.93	1.75	0.52	0.013
Ti	0.47	0.82	0.47	0.45	0.24	0.042
V	0.04	0.07	0.03	0.03	0.03	0.100
Zn	98.50	138.60	96.80	98.40	16.52	<.001

4.4 Discussion

4.4.1 Honeybees

Forager bees are good samplers (Satta et al., 2012). In the current study in-hive bees were sampled. In-hive bees taken from the outer frame of the brood box represent the average bee in the colony (Steen et al., 2012[b]). In a hive, particles on the bee's body exchanged via physical contact (Degrandi-Hoffman et al., 1984; Free & Williams., 1972; Paalhaar et al., 2008) and in the nectar via trophallaxis. Following the objective of the study: collect data on the spatial variation of metal concentrations in honeybee colonies in the Netherlands, sampling of in-hive bees and subsequent pooling of the bees per apiary was preferred over sampling of forager bees of individual hives per apiary. This study was a surveillance

study to record spatial variation and not a bio-indication study focussed on a possible explanation of differences of metal concentration in bees.

4.4.2 Metal concentration

Based on the trend line that can be drawn in the figures in Figure III, Al, Ba, Cr, Mn, Mo and Se are present in higher concentrations in the eastern part of the Netherlands whereas As shows the opposite. Cd, Co, Cu, Li, Ni, Sb, Sn, Ti, V and Zn show a horizontal trend line over the regions showing no higher concentrations in bees in the East or the West of the Netherlands. Figure III reveals regional patterns. Generally, per metal and per region, the concentrations above or below the median are clustered. Relatively high concentrations of Al, Ba, Cr, Cu, Mo, Ni and Zn are concentrated in the region roughly bordered by the cities Arnhem. Apeldoorn, Enschede and Winterswijk. Se shows two regions with high values: the region Zuid Holland and the region Oost Overijssel, Drenthe, Friesland and Groningen. All apiaries are ranked in ascending postal code numbers up to the four numbers and the bars in figure II represent apiaries in each other's vicinity within the specific postal code region. More in detail it can be seen that for all metals in-region concentration varies, showing a local effect. In praxis this means for heavy metal studies with honeybees: "think regionally, act locally". Besides spatial variations also temporal variations have been reported (Steen et al., 2012[a]). The current study has been performed once. Studying metal concentrations in bees in the Netherlands in another time of year might give a different outcome.
As shown, metals are present in honeybees in a broad concentration range. It is the result of actual presence of metals in the food (pollen, nectar, honeydew and water), biological presence of metals in bee's body plus possibly metals deposited in the flowers from atmospheric deposition of metal containing particles. The findings indicating significant differences in exposure ranging from low to zero exposure up to high exposure. In a previous study on spatial and temporal variation of metal concentrations in adult honeybees (Steen et al., 2012[a]) concentrations exceeding significantly the mean ($P \leq 0.05$) were considered to indicate a higher exposure. In bio-indication studies by Porrini et al. (2002[a]) and Gutiérrez et al. (2015) high (upper quartile > 75 percentile) and low (lower quartile 25 percentile) reference thresholds (Table 4) are applied based on study results in Italy (Porrini et al.) and Spain (Gutiérrez et al.). In these studies, concentrations above the 75 percentile quartile were considered to be worrisome. The Ni, Cr and Cd data recorded in the current study are within the safe range according to Porrini et al. (2002[a]). Cr recorded in the current study would be worrisome, taking the high and low reference thresholds set by Gutiérrez et al. (2015).

Table 4. High and low reference thresholds in mg.kg wet matter (wm) bee^{-1}

	Porrini et al., 2002	Gutiérrez et al., 2015	Current study*
Pb	0.40 – 2.0	0.3 – 0.7	
Ni	0.10 – 0.40	0.1 – 0.3	0.13 – 0.24
Cr	0.04 – 0.25	0.04 – 0.12	0.11 – 0.17
Cd		0.052 – 0.1	0.05 – 0.10

* The data of the current study presented in table 2 in dry matter bee (dm) are converted to wet matter (wm) bee taking into account the weight loss of the drying process of 68% (Steen et al., 2012[a])

As shown in Table 4, high and low reference values differ significantly per study, demonstrating the broad range of concentrations of heavy metals in honeybees. This variation is both temporal and spatial and therefore only applicable under defined conditions (Steen et al., 2012[a]). The current surveillance study implies only spatial variation as the samples were taken in a limited time window of about one week in June 2008. Compared to the mean concentrations of metals in µg metal.dm honeybee^{-1} in the Steen et al., 2012[a] study conducted in 2006 at three locations, the metals Al, Cr, Mn, Ni, Sb, Se, Ti and Zn show higher mean concentrations but are all, except Cr and Mn, within the 95% probability area of metal concentrations in the current study (mean + 1.66 x sd, one-sided). Compared to previous reported reference data (Steen et al., 2012[a]) the mean concentrations of As, Cd, Cr, Cu, Mn, Ni, Se and Zn recorded in the current study are all except Mn and Ni in the same range as detected in reported control sites (Bromenshenk et al., 1985; Veleminsky et al., 1990; Fakhimzadeh & Lodenius, 2000; Porrini et al., 2002[a]; Roman, 2005). The Mn and Ni concentration exceeded the reported concentrations (Kump et al., 1996; Porrini et al., 2002[a]; Roman, 2005). The concentration ranges published and in the current study, show for each metal a large variation. Based on the demonstrated regional differences in the current study, threshold limits should be set per region and land use should be taken into account (see Land use). Hives in the same apiary show different metal concentrations in the bees (Steen et al., 2012[a]). Sampling multiple colonies per apiary provide a better overview of foraging sites within the foraging area of a apiary. Bees of colonies in one apiary divide themselves of the foraging area (Waddington et al., 1994). Therefore, pooled apiary samples can do for this type of surveillance study

4.4.3 Land use

The selection of the apiaries was not directed by land-use but by the requirement of an overall coverage of apiaries over the Netherlands. As shown in Table 3 the majority of the apiaries sampled are in agricultural areas, next in mixed land use areas, then in urban areas and at the rear end wooded areas. Despite the low numbers of apiaries in > 50% wooded areas, statistically significantly higher concentrations were recorded there, indicating the impact of the land use on metal concentrations in the bees. This phenomenon was also observed by Lambert et al. (2012[b]). Further studies on the impact of land use should be done to reveal the mechanisms. Non comprehensive reflections on why bees in wooded areas have higher concentrations metals are that at wooded sites atmospheric deposition is greater to a forest interior than to a forest edge (Fowler et al., 2004). This may be due to decreasing wind speed in wooded areas (Raynor et al., 1974; Pleijel et al., 1996). Additionally, trees promote vertical transport by enhancing turbulence (McDonald et al., 2007). Honeydew resulting in sticky leaves is assumed to be more prevalent in wooded areas than others, possibly resulting in an increased physical binding of metal containing particles from atmospheric deposition (personal communication dr R. Moosbeckhofer). In general dark honeys contain honeydew and have higher metal concentrations compared to light honey (Crane, 1979). These typical features of a wooded site may affect increased deposition of airborne metal containing particles, originating from other locations. Measuring metal concentrations in honeybees for bio-indication purposes is an indirect recording of the sum of metals in pollen, nectar and honeydew plus possibly additional deposition. This sum can't be split in the two terms as deposition of heavy metals is not recorded separately. This is the intrinsic uncertainty of heavy metal bio-indication studies with honeybee colonies. Elevated concentrations of specific metals are always the result of higher exposure, but this does not need to be one to one related to the level of deposition.

4.5 Conclusion

Honeybee colonies proved to be applicable as bio-indicator of metal burden in the regional and local environment. Honeybees in apiaries in different regions in the Netherlands have different concentrations metals, a specific regional effect. Within the regions are local differences. The data indicate higher metal concentrations in > 50% wooded areas, compared to > 50% agricultural, > 50% urban and mixed used areas, a local effect. For qualitative bio-indication studies, regional and local effect should be taken into account. Furthermore, land use effect should be studied in

detail to reveal the mechanisms resulting in different concentrations of metals in bees. Both regional and local effects have consequences for conclusions on overexposure of bees to metals in comparison studies.

Acknowledgement
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Chapter 5

"The perfect match" Crop pollination and bio-indication of plant pathogens by honeybee colonies

J.J.M. van der Steen; M. Bergsma-Vlami, W.J. de Kogel & M. Wenneker. This chapter is submitted for publication.

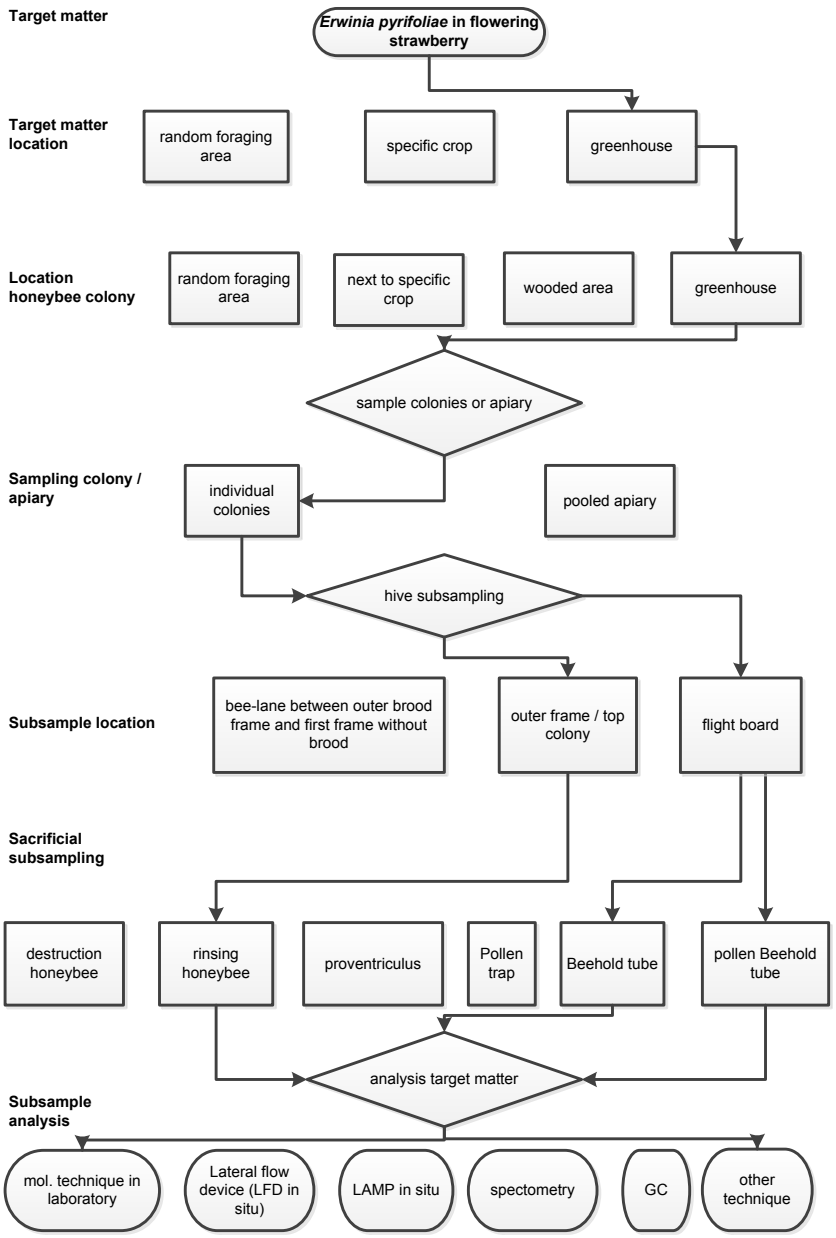

Figure I. Bio-indication flow chart:
The perfect match
Crop pollination and bio-indication of plant pathogens by honeybee colonies

Abstract
In this study we show that the honeybee colonies placed in a greenhouse for pollination of strawberry can simultaneously be used to indicate the presence of the plant pathogen *Erwinia pyrifoliae*. This was demonstrated using two methods of qualitative bio-indication: sacrificial and non-sacrificial subsampling. The non-sacrificial subsampler Beehold device was applied. In the Beehold device, hive-entering and hive-leaving bees are separated. Hive-entering bees are forced to enter the hive via a tube, internally lined with polyethylene glycol (PEG). The study demonstrated that the integration of pollination and bio-indication matches. In both sacrificial and non-sacrificial derived subsamples *E. pyrifoliae* was detected. *E. pyrifoliae* was detected prior to occurrence of *E. pyrifoliae* symptoms in the flowers. The Beehold tube is a practical tool for monitoring plant pathogens via forager bees during flowering until fruit onset.

5.1 Introduction

Honeybee colonies (*Apis mellifera* L) are widely applied for pollination. Honeybee (cross) pollination is the result of (1) collection of pollen and nectar in the flower, (2) flower constancy at a foraging trip, and (3) in-hive exchange of pollen, resulting in a passive pollen load of all bees with pollen of diverse botanic origin, both in-hive and forager bees (Degrandi-Hoffman et al., 1984). During food collection in flowers and occasionally on the leaves, pollen and non-floral particles such as plant pathogenic bacteria and atmospheric deposited particles, adhere to the branched hairs of the honeybees. The major part is removed by auto-grooming during collection and in-hive allo-grooming. However, part of the particles remains on the bee's exterior (Free & Williams, 1972). The honeybee's feature of unintentionally collection of plant pathogenic bacteria, both stand-alone and attached to pollen, makes each foraging honeybee an applicable in-flower plant pathogen micro-sampler. For bio-indication, the honeybee colony is considered to be the sampling tool which is next subsampled to detect plant pathogenic bacteria or other non-floral particles on or in the bees. Although pollination and bio-indication is a logical match, it is not common practice. Bio-indication of the plant pathogenic bacterium *Erwinia amylovora*, the causal agent of Fireblight, is an exception in this and practiced in Italy, Austria and Switzerland (Halbwirth et al., 2014; Porrini et al., 2002[b]).

Subsampling of the honeybee colony can be done sacrificially or non-sacrificially. Sacrificial subsampling means taking bees from the colony and killing the bees for analysis (destructive sampling). Considering the honeybee's performance, sacrificial subsampling has its practical limits regarding frequency and sample size. Every honeybee taken weakens the colony to some extent. In contrast, non-sacrificial subsampling (non-destructive sampling) does not go at the expense of the honeybee colony. The goal of non-sacrificial honeybee subsampling is to remove physically part of the particles from the hive-entering bee's exterior without removing honeybees from the colony. The number of bees for non-sacrificial sampling is unlimited and one single bee can be sampled multiple times. In Austria the non-sacrificial subsampler, applied for bio-indication of *Erwinia amylovora* in flowering fruit trees, is a tube internally lined with a plastic sheet (Halbwirth et al., 2014). In the current study, the Beehold tube is applied. The Beehold tube is an innovative non-sacrificial subsampler concept, developed by the corresponding author at Plant Research International, Wageningen UR, The Netherlands. *Erwinia pyrifoliae* is a recently described bacterial disease of strawberry causing reduction of the production of strawberry under greenhouse cultivation conditions. Symptoms include brown petals, green young fruits

turning brown, malformed fruits and bacterium slime (ooze) formation on the surface of the young fruits (Wenneker & Bergsma–Vlami, 2015). Sacrificial and non-sacrificial subsampling of the honeybee colony to detect *E. pyrifoliae* in flowering strawberry greenhouse cultivation has been tested in an experimental trial. By regular subsampling honeybee colonies from the start of the blooming period till the post blooming period, *E. pyrifoliae* infection prior to symptoms development on the plants might be detected. Applying honeybees for bio-indication results in a qualitative outcome, a signal for further more specific study.

5.2 Material & Method

The bio-indication scheme is presented in the flow chart on page 90 (Figure I).

5.2.1 Study site, honeybee colonies and study period

The study has been conducted in a four-hectare greenhouse in Made (Province Noord-Brabant, The Netherlands) planted with strawberry (*Fragaria* x ananassa, cultivar Elsanta). The honeybee colonies were obtained from apiary Ecopol Geffen (Province Noord-Brabant, The Netherlands). The observation period started on March 10th, 2015 and ended on April 15th, 2015.

5.2.2 Subsampling of the honeybee colony

On March 14th, at the start of the blooming of the strawberry plants, six honeybee colonies were placed in the greenhouse for pollination. Prior to the translocation of the colonies from the apiary to the greenhouse, 30 hive-entering bees were taken from one honeybee colony at March 10 and tested for the presence of *E. pyrifoliae*. After translocation, the first and last colony in the line of the hives in the greenhouse was marked for both sacrificial and non-sacrificial subsampling. In the entrance of the two colonies, the non-sacrificial subsampling Beehold device, including a bee counter was inserted. Subsampling continued during the entire flowering period according to Table 1. To ensure sufficient pollinating honeybees, the marked honeybee colonies were replaced at April 3th, by 'fresh' colonies from the same apiary. Ergo, samples taken at March 18th, 25th and April 1st were from the same colonies, and the April 8th and 15th samples were taken from newly introduced colonies. The newly introduced colonies were not tested for the presence of *E. pyrifoliae* prior to translocation into the greenhouse.

Table 1. Sampling dates, exposure periods, colony id and sample id of the 2015 study

Sampling date	Activity	Exposure period	Location	Sacrificial / non-sacrificial subsampling	Colony id	Sample id
			Honeybees			
10 March	Bees sampled		Apiary Geffen	Sacrificial	Col 1	Bij 1
16 March	Beehold tubes inserted		Greenhouse			
18 March	Beehold tubes replaced	16 + 17 March	Greenhouse	Non-sacrificial	Col 1	Bij 6
18 March	Beehold tubes replaced	16 + 17 March	Greenhouse	Non-sacrificial	Col 2	Bij 7
25 March	Bees sampled		Greenhouse	Sacrificial	Col 1	Bij 10
25 March	Bees sampled		Greenhouse	Sacrificial	Col 2	Bij 11
25 March	Beehold tubes replaced	18 - 24 March	Greenhouse	Non-sacrificial	Col 1	Bij 13
25 March	Beehold tubes replaced	18 - 24 March	Greenhouse	Non-sacrificial	Col 2	Bij 14
1 April	Bees sampled		Greenhouse	Sacrificial	Col 2	Bij 17
1 April	Beehold tubes replaced	25 – 31 March	Greenhouse	Non-sacrificial	Col 1	Bij 20
1 April	Beehold tubes replaced	25 – 31 March	Greenhouse	Non-sacrificial	Col 2	Bij 21
8 April	Bees sampled		Greenhouse	Sacrificial	Col 3	Bij 24
8 April	Beehold tubes replaced	1 – 7 April	Greenhouse	Non-sacrificial	Col 3	Bij 27
8 April	Beehold tubes replaced	1 – 7 April	Greenhouse	Non-sacrificial	Col 4	Bij 28
15 April	Bees sampled		Greenhouse	Sacrificial	Col 3	Bij 31
15 April	Beehold tubes replaced	8 – 14 April	Greenhouse	Non-sacrificial	Col 3	Bij 34
15 April	Beehold tubes replaced	8 – 14 April	Greenhouse	Non-sacrificial	Col 4	Bij 35

5.2.3 Sacrificial subsampling of the honeybee colony

Sacrificial subsampling was performed weekly by randomly picking 30 bees from the top bars of the frames just below the cover board of the hive. The bees were directly put in a 50 ml Greiner blue cap tube filled with 20 ml phosphate saline buffer (PBS 10 mM, pH 7.2) and transported to the laboratory within two hours.

5.2.4 Non-sacrificial sub-sampling of the honeybee colony with the Beehold tube

The Beehold tubes, the sampling part of the Beehold device, were according to the sampling scheme, replaced weekly by new ones in the morning prior or at the start of the colony's activity. The exposure period of the hive-entering bees to the Beehold tube is the period hive-entering

bees pass the Beehold tube. Because of the replacement prior to the colony's activity, the exposure period of each Beehold tube ends the day prior to the sampling day. After removal, the Beehold tubes were directly put in a sterile Greiner blue cap tube of 50 ml, transported to the laboratory and elaborated within two hours after collection.

The Beehold device, schematically presented in Figure II, is a non-sacrificial subsampler of honeybee colonies in which hive-entering and hive-leaving bees are forced to leave and enter the hive via different tubes. It consists of a foam strip that seals off the complete hive entrance except minimally two openings, one for the Beehold tube and one for the out-tube. The Beehold tube, 11 cm long and with an inner diameter of 1.9 cm, is internally covered by a thin transparent PVC foliar holding a sticky polyethylene (PEG) layer, covered with plastic gauze to enlarge the surface and stabilize the PEG's position on the PVC layer. The moderate sticky PEG layer adheres physically part of the particles attached to the hive-entering bee's hair and feet. The stickiness of the PEG depends on the ambient temperature. For this study a mixture of one part PEG1000 and one part PEG1500 (v/v) was applied. PEG is non-toxic to bees and can be applied safely for study objectives (Crailsheim, 1985). An accustomed bee counter, attached to the Beehold tube, recorded the number of hive-entering bees. To calculate the detectable minimal mean number of plant pathogens a bee must collect daily, the Beehold formula was applied. In this formula the terms are: (1) minimal detectable amount (Limit of Detection LOD) of the analysis protocol; (2) number of bees that passed the Beeholdtube per day; (3) the minimal theoretic adsorption rate (fraction) of matter from the bee's body to the PEG (0.01); (4) the assumed fraction of particles left on the bee's body after auto-grooming during foraging and the return flight plus part of the pollen in the corbicula (0.02) and (5) the fraction of the bees that foraged on the target crop based on the ratio between pollen from different botanic origin.

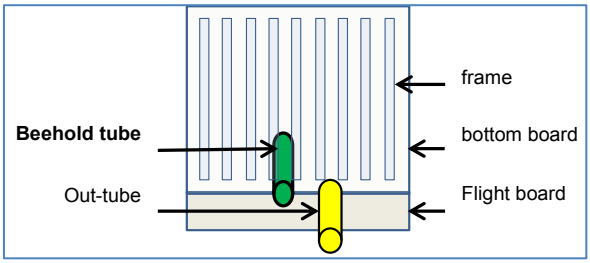

Figure II. The Beehold device.
Schematic top-down drawing of the position of the Beehold tube and out tube on the flight board and bottom board. of the hive

Beehold formula

$$SCR\ \min = \frac{LOD}{n} * \frac{1}{F\ \min\ * F\ left}$$

Herein are:

1. SCR min = specific minimal collection ratio, i.e. Minimal number of *Erwinia pyrifoliae* bacteria a bee should collect per day to accumulate a detectable amount γ-HCH in the Beehold tube (number.day^{-1});
2. LOD, minimal detectable amount (LOD analysis protocol); (number)
3. n, number of bees passing the Beehold tube per one day (n.period^{-1});
4. F min, the minimal theoretic adsorption rate (fraction) of matter from the bee's body to the PEG in the Beehold tube = 0.01 (paragraph 5.4.2);
5. F left, the assumed fraction of particles left on the bee's body after auto-grooming during foraging and the return flight = 0.02 (paragraph 5.4.2).

The "Protocol Beehold tube" describing step by step the Beehold tube method from preparation till analysis is available from the corresponding author.

5.2.5 Preparation of the samples for detection of *E. pyrifoliae* and functionality check of the Beehold tube

In the laboratory, the sacrificially derived 30-bees samples were mechanically shaken for minimally two minutes, suspending particles from the bee's exterior into the 20 ml phosphate saline buffer (PBS 10 mM, pH 7.2) in which the bees were collected. Before shaking, some droplets Tween 80 were added to facilitate removal of particles from the hairs. Next, an aliquot of 500 µl buffer was pipetted in a 1.5 ml Eppendorfer tube for pollen determination. An aliquot of 12 ml was pipetted into a sterile 12 ml sealable tube and transported immediately to the laboratory. From the non-sacrificially derived Beehold tubes, the PVC layer with the PEG and gauze was removed from the Beehold tubes and inserted in the Greiner 50 ml blue cap tube in which the Beehold tube were transported. In this tube 1.5 ml phosphate saline buffer was pipetted plus some droplets of Tween 80. To dissolve the PEG into de buffer, the blue cap tubes were horizontally placed in a rotator and rotated for minimally 15 minutes at room temperature. An aliquot of 500 µl of the PEG/phosphate

buffer mixture was taken for check of the functionality of the Beehold tube and for pollen identification. The remainder of the PEG/phosphate buffer mixture was pipetted into 12 ml sterile tubes and transported immediately to the laboratory.

5.2.6 Pollen determination / functionality of the Beehold tube

Presence of pollen proves the functionality of the Beehold tube defined as adherence of particles, including pollen, from the bee's exterior to the PEG layer. The botanic origin of the pollen reveals if and where the foragers collected their feed. The Eppendorfer tubes with the 500 µl rinsing fluid of the honeybees of the sacrificial sampled bees and the mixture PEG/phosphate buffer were centrifuged for 10 minutes at 14000 rpm to concentrate the pollen. After centrifuging, the aliquot was poured off and the remaining pellet was re-suspended in the remaining approximately 40 µl supernatant. Next 10 µl of the supernatant was pipetted on a microscope slide, dried at 70 °C on a temperature controlled heater, covered with fuchsine stained gelatin/glycerine (Kaiser), sealed with a microscope cover glass and stored at room temperature till microscopical determination.
The botanical origin of the pollen was determined by morphological characteristics of 100 pollen grains (Hodges, 1974; von der Ohe & von der Ohe, 2001). The ratio *Fragaria x ananassa* (strawberry) pollen and pollen from other plant species was calculated.

5.2.7 Detection *Erwinia pyrifoliae*

Recovery and population size of *E. pyrifoliae* in honey bee body were determined by dilution plating on YPG (Yeast Peptone Glucose) agar medium. A 20 µl aliquot of extract was streaked by dilution plating on three plates of the medium. Plates were incubated for 2 to 3 days at 28°C. The isolation was negative if no bacterial colonies with morphology similar to *E. pyrifoliae* were observed after 96 h and that typical *E. pyrifoliae* colonies were found in the positive control. Pure cultures of presumptive *E. pyrifoliae* isolates were identified with the specific molecular test according to Wensing et al. (2011). The lowest detectable number of *E. pyrifoliae* (LOD) in the PCR protocol applied is 100 cells per reaction.

5.3 Results

5.3.1 Pollen / Beehold tube functionality

The hive-entering bees, sampled sacrificially prior to translocation from the apiary to the greenhouse, did not carry strawberry pollen, demonstrating the bees did not forage on strawberry prior to placement in the greenhouse. The colonies arrived in the greenhouse on March 16th and

first time sampled on March 18th. The March 18th samples did not contain strawberry pollen, showing the bees had not yet started to forage on the strawberry flowers in the greenhouse. A week later the in-hive bees taken from the top bars, did not contain strawberry pollen but the Beehold tubes did, demonstrating foraging activity on the strawberry flowers. From April 1st, all samples contained almost exclusively strawberry pollen demonstrating the bees foraged exclusively on strawberry. Both mature and dry state of *Fragaria x ananassa* pollen was present which is not abnormal in strawberry pollen (Dafni et al., 2012). Strawberry pollen in the Beehold tubes demonstrated hive-entering bees passed the Beehold tube, and therefor proving its functionality.

5.3.1 *Erwinia pyrifoliae*

No *E. pyrifoliae* was detected on the hive-entering bees of the colony sample taken prior to placing the honeybee colonies in greenhouse.

5.3.2 *Erwinia pyrifoliae* on sacrificial subsampled in-hive honeybees

E. pyrifoliae was detected two weeks after the introduction of the colonies in the greenhouse in one of the two March 25th samples (sample id Bij11, colony 2). This result showed that in the period March 10th till March 25th, *E. pyrifoliae* became present in detectable levels on the flower's surface. In the April 1st samples, *E. pyrifoliae* was again detected on in-hive bees from colony 2 (sample id Bij17). In both the April 8th and April 15th samples, in the newly placed sampled honeybee colony (colony 3), *E. pyrifoliae* was detected.

5.3.3 *Erwinia pyrifoliae* in non-sacrificial Beehold tube samples

In the March 18th and March 25th samples no *E. pyrifoliae* was detected. In the April 1th samples, one Beehold tube (sample id. Bij20, colony 1) contained *E. pyrifoliae*. In the Beehold tube of colony 2 no *E. pyrifoliae* was detected then. In the April 8th samples in both Beehold tubes from colony 3 and 4, *E. pyrifoliae* was detected. In the April 15th sample, one Beehold tubes (colony 3) had *E. pyrifoliae* and the other from colony 4 not.

The overall results (positive / negative in minimally one of the matrixes) of the *E. pyrifoliae* detection on the in-hive bees and in the Beehold tubes are presented in Figure III.

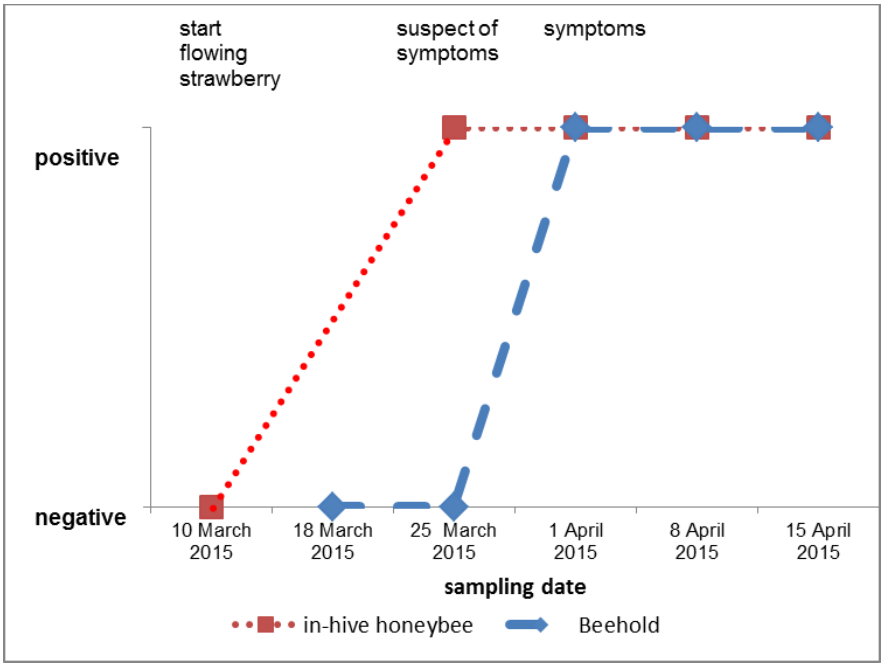

Figure III. Detection of *Erwinia pyrifoliae* on the honey bees, and in the Beehold tube. On top of the figure the start of the flowering, the period the *E. pyrifoliae* infection was suspected and the start of the period the *E. pyrifoliae* infection was clearly visible in the crop. The location of the textboxes corresponds with the exposure period between the sampling dates presented on the X axis.

5.3.4 Number of hive-entering honeybees passing the Beehold tube

On average 75 (minimum 22, maximum 132, n = 4) honeybees passed the Beehold tube daily. Due to a technical failure, the number of hive-entering bees via the Beehold tube was not counted continuously. The data presented are the mean of 7-days counts. The number of hive-entering bees is low. The four hectares foraging area is relatively small compared to the 2800 hectares potential foraging area of a honeybee colony in the field.

5.3.5 Minimum number of cfu *E. pyrifoliae* collected by the foragers to be detectable in the Beehold tube

On average, each bee collected minimally 6667 cfu's *E. pyrifoliae* in a strawberry flower per day.
The terms of the Beehold formula were:

- LOD analysis method: 100
- # bees passing the Beehold tube per day: 75
- ratio matter assumed adhered to the Beehold tube: 0.01 (1%)
- ratio matter left after self-grooming: 0.02 (2%)
- ratio bees that foraged on the target plant: 1

5.4 Discussion

5.4.1 Sacrificial subsampling

Sacrificial subsampling of the honeybee colony is at the expense of the colony's performance. It is assumed, based on long-year practical experience and inter-collegial discussions, that minimally 1.5% of the in-hive bee cohort can be sampled safely in a 3-week period. The honeybee colonies in the greenhouse contained 4000 to 6000 bees. Consequently, 60 to 90 bees could have been taken from the colony. The applied sample size of 30 bees meets the safety threshold. In-hive physical exchange of particles on the bee's exterior and trophallaxis goes within days (Nixon & Ribbands, 1952). This exchange pertains the in-hive sacrificial sampling. The number of *E. pyrifoliae* per in-hive bee depends on the influx of *E. pyrifoliae* collected by the forager bees. Applying the binomial probability theory equation N=ln(1-D)/ln(1-P) in which N is the sample size, D is probability of detection (power) and P is the minimal portion of bees carrying target matter, the chance at least two bees (fraction 0.075) will carry *E. pyrifoliae* in a 30 bees sample is 90%. The longevity of *E. pyrifoliae* on the honeybee's exterior is not known. The viability on the honeybee of the related bacterium *Erwinia amylovora* is up to two days (Wael, 1988). Assuming the same survival period of *E. pyrifoliae*, the detection of *E. pyrifoliae* on 24th March indicates the foragers collected the bacterium from 22nd March. As the first suspect of an *E. pyrifoliae* infection by the grower (ooze droplets) was in the period March 25th - April 1st, the honeybees collected *E. pyrifoliae* prior to the observation of any symptoms of an infection in the strawberry crop. In one of the two sampled colonies *E. pyrifoliae* was detected. This may be the result of separate or interacting features as there are: (1) the bees of this colony visited more infected flowers than the other sampled colony, (2) the honeybees of the colonies in the greenhouse were not homogeneously

dispersed over the flowers, (3) the presence of *E. pyrifoliae* started locally, and (4) little inflow of cfu *E. pyrifoliae* which was diluted by in-hive exchange to a non-detectable number. On April 3th, new honeybee colonies were placed in the greenhouse. The first sampling of these colonies was in April 8th. In the April 8th sample, *E. pyrifoliae* was detected showing that within 4 days sufficient cfu *E. pyrifoliae* entered the hive and next exchanged within the colony to be detectable.

5.4.2 Non-sacrificial subsampling with the Beehold tube

Particles from forager bee's exterior, entering the hive via the Beehold tube, adhered to the PEG. The detection of *E. pyrifoliae* in the PEG confirmed its functionality as non-sacrificial bio-indicator tool. The outcome of the Beehold formula of on average 6667 cells of *E. pyrifoliae*, showed that this number was minimally collectable per forager bee per day. The Beehold tube is a qualitative non-sacrificial sampler which bio-indicated the presence of *E. pyrifoliae* in the crop qualitatively.

During foraging, bees clean themselves by auto-grooming and in hive by allo-grooming resulting in a fraction of pollen and un-intended collected particles in the bee's hairs and foot parts. The majority of the pollen is accumulated during foraging in the pollen baskets (*corbicula*) on the bee's hind legs. Paalhaar et al. (2008) demonstrated that honeybees that never left the colony have pollen in the hair and that relatively small grains are dominant. Based on the permanent passive load of pollen in the bee's hairs of 4000 to 13000 pollen grains of bee leaving the hive (Free & Williams, 1972) and the estimated number of pollen grains collected during a pollen foraging trip of 153000 to 30000 grains (average weigh of a pollen load is 15 – 20 mg; average weight of a pollen grain is 50 – 100 ng (Kleinjans et al., 2012; Babendreier et al., 2004), it is assumed 2 – 4% of particles collected, remain on the bee's exterior. In the Beehold formula, a fraction of 0.02 (i.e. 2%) is applied as term of fraction particles remaining on the bee's exterior after grooming.

Adherence of particles from the bee's exterior to the PEG was preliminarily studied in the laboratory with charcoal particles and in a semi-field trial with the plant pathogen *Erwinia amylovora*. The mean adherence of charcoal particles was 51% (sd 25%, n: 58). Applying the 95% probability limits (one sided), the 95% threshold is 9% (mean-1.66*sd) in other words, under laboratory conditions there is 95% probability, minimally 9% of the particles adhere to the PEG. It must be mentioned that in the laboratory test set-up the bees had no optimal condition for flying and auto-grooming. The semi-field trials with *E. amylovora* resulted in 2% adherence from the bee to the PEG. In the Beehold formula the fraction of 0.01 (1%) is applied.

The split-up of hive-entering and hive-leaving bees is derived from the known beekeepers' method, applied to allow only hive-entering bees to come in and prevent hive-leaving bees to leave the hive. The method uses the honeybee's feature that hive-entering bees approach the hive entrance via landing on the flight board or on the outer front board and next walking towards the entrance. Hive-leaving bees exit the hive via a walk on the bottom board or via the inside front wall or the hive towards the flight board to find their way out via an opening in the front board of the hive. The Beehold tube protrudes the inner front wall of the hive to prevent hive-leaving bees to exit the hive via the Beehold tube. To prevent hive-entering bees to enter via the out-tube, the out-tube protrudes the flight board.

5.4.3 Pollen in Beehold tubes

Before the honeybee colonies were placed in the greenhouse, no strawberry pollen was detected on the bees. In the 2015 study during the first two days after placement in the greenhouse, no pollen was recorded in the Beehold tubes. On the bees no strawberry pollen was recorded then. From some days after the start of the observations till end of blooming in most honeybee colonies 100 % strawberry pollen was recorded. At some occasions little pollen was found in the Beehold tube after a week exposure. These colonies found an alternative way to enter the hive instead of passing the tube.

The pollen data show that the honeybee colonies had not foraged on strawberry prior to translocation in the greenhouse and once in the greenhouse, forager bees visits solely strawberry flowers in the greenhouse.

5.4.4 Early detection *Erwinia pyrifoliae* by sacrificial and non-sacrificial subsampling

Sacrificial subsampling of in-hive bees was more accurate compared to the Beehold tube. *E. pyrifoliae* was detected on in-hive bees prior to any symptoms of an *E. pyrifoliae* infection. In the period prior to the any visible symptoms, *E. pyrifoliae* was collected by the foraging bees in numbers detectable on the in-hive bee cohort and not detectable in the Beehold tube. With the Beehold tube, *E. pyrifoliae* detection coincided with the first visible symptoms of the infection. Minimally two of the in-hive 30 bee-sample carried *E. pyrifoliae*. These two bees may have happened to be foragers just returning from a foraging trip. However, it is more plausible that the *E. pyrifoliae* bacteria on in-hive bees were the result of in-hive exchange from foragers to the in-hive bee cohort. The calculated minimum required number of 6667 *E. pyrifoliae* bacteria per hive-entering bee per day for non-sacrificial subsampling, appeared not to be met in the

period between first presence of *E. pyrifoliae* and the appearance of the first tiny bacterium slime droplets. The adherence of the bacterium to the PEG appeared to be insufficient to accumulate *E. pyrifoliae* in a detectable number at this early stage of the infection. To improve non-sacrificial subsampling for bio-indication of *E. pyrifoliae* prior to the appearance of the ooze droplets, further study must be done to improve the adherence of bacteria to the PEG and the intensification of the contact between PEG and hive-entering bees. It was striking that flowers showing symptoms of the *E. pyrifoliae* infection are next to flowers not showing symptoms. The grower had no precise assessment of the percentage infected flowers, but estimated the percentage infected flowers on April 15th at approximately 10%.

5.5 Conclusion

The integration of pollination and bio-indication by a honeybee colony matches. Both sacrificial- and non-sacrificial subsampling of honeybee colonies can be applied for qualitatively bio-indication of *E. pyrifoliae* in strawberry greenhouse cultivation during flowering. *E. pyrifoliae* was detected prior to visible symptoms of the infection in the flowers applying sacrificial subsampling of in-hive bees. Detection of *E. pyrifoliae* by non-sacrificial sampling with the Beehold tube coincided with the first visible symptoms of the *E. pyrifoliae* infection in the flowers. Non-sacrificial subsampling with the Beehold tube can be done by the strawberry growers themselves, providing a practical tool for monitoring plant pathogens. Future development of an in situ detection of *E. pyrifoliae* can help the growers themselves to monitor the presence of pathogens in the crops with the pollinating insects.

Acknowledgement
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Chapter 6

Bio-indication of *Erwinia amylovora* in flowering fruit orchards in Austria (Steiermark) with the non-sacrificial subsampler Beehold tube

J.J.M. van der Steen. 2015.
Study report Plant Research International, Wageningen UR (in preparation)

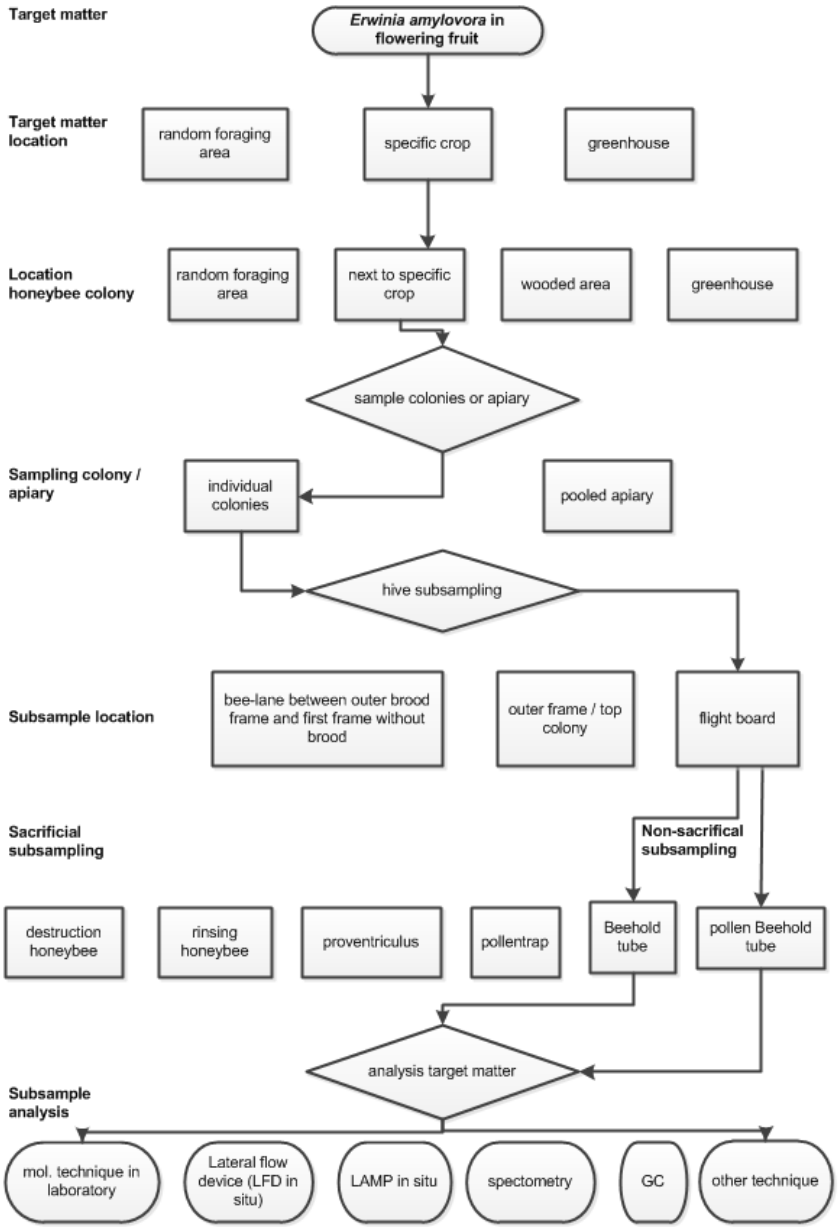

Figure I. Bio-indication flow chart: Bio-indication of *Erwinia amylovora* in flowering fruit orchards in Austria (Steiermark) with the non-sacrificial subsampler Beehold tube

Abstract
In the period 29th April – 10th May 2013 the non-sacrificial sampler Beehold tube was applied to subsample the honeybee colony for the detection of *Erwinia amylovora* (Burrill) in a field study in three flowering fruit orchards in Steiermark, Austria. The Beehold tube is part of the Beehold device. In the Beehold tube hive-entering bees pass a tube, internally covered with a PVC sheet with a thin layer polyethylene glycol (PEG), a water soluble moderate sticky material, meant to adsorb, particles attached to the honeybee's hairs and foot parts. In preliminary tests, the Beehold tube functioned in adsorbing detectable amounts of *Erwinia amylovora*. For comparison of the functionality of the Beehold tube, the study was done simultaneously in the same apiaries with other colonies, with the proven functioning non-sacrificial sampling with Caledonia NVG25 sheets, developed by Dr Moosbeckhofer and applied by AGES Austria. No *Erwinia amylovora* was detected, nor in the Beehold tube nor in the AGES samples nor in the orchards. It can be concluded that the prevalence of *Erwinia amylovora* in the orchards, tested in spring 2013 was low to nihil. It can be concluded that based on the Limit of Detection (LOD) of 2 cfu *Erwinia amylovora* in 1 µl PEG / water suspension, analysed with qPCR, the number of bees that passed the tube and the minimal adsorption rate of the Beehold tube, each bee that passed the Beehold tube carried < 1.15E+5 cfu *Erwinia amylovora*.day^{-1}. As there was no fireblight infection in Steiermark in spring 2013, the functionality of the Beehold tube to detect *E. amylovora* could not be confirmed in this field trial. The presence of *Rosaceae* pollen showed the bees bio-sampled in the orchards.

6.1 Introduction

Erwinia amylovora (Burrill) causes fireblight, an infectious disease of plants of the *Rosaceae* family. The bacterium hibernates in infected plants. In spring the germination process starts and a primary inoculum is formed. This inoculum can be disseminated by insects, birds, rain and wind. The portes d'entrée of plants are the natural orifices in flowers, the stigma and nectaries (Wael, 1988; Pusey, 2000). Pusey & Curry (2004) studied *E. amylovora* (Ea153) development on stigma, a natural porte d'entrée of epiphytic *E. amylovora*. It was demonstrated that the temperature for *E. amylovora* multiplication ranges from 8 to 36 ^{0}C with an optimum ranging from 20 to 32^0 C. The age of the stigma for optimal bacterium growth decreases with increasing temperature and successful pollination decreases bacterium growth on the stigma. Increasing relative humidity stimulates bacterium growth (Pusey, 2000). Ivanoff & Keitt (1941) found that the sugar concentration in the nectar determines whether the bacterium can germinate; the higher the sugar concentration the slower the germination process, the optimal sugar concentration was 2-4%. At > 30 % sugar no bacterium germination was recorded. Bacterium growth on the anthers contaminates the pollen (Bubán & Orosz-Kovács, 2003). In summary, for favourable conditions for *E. amylovora* to cause a fireblight infection, a warm period in which the bacterium can be disseminated by wind, birds and insects, followed by a period of low temperatures, high humidity and non-optimal pollination prolongs the vulnerable period for successful *E. amylovora* infection and increases the chance of a fireblight infection. Depending on temperature, nectar sugar concentration and humidity it can take up to days before clinical signs on the trees can be observed (Pusey, 2000).

Honeybees are specialised in collecting pollen and nectar. Along with this collection process, matter present in and on flowers like bacteria, are collected unintentionally. By checking bees, pollen and nectar for the prevalence of *E. amylovora*, the honeybee is a potential tool to detect fireblight. Wael, 1988; Alexandrova et al., 2002 and Porrini et al., 2002[b], demonstrated that *E. amylovora* is transferred from infected flowers to non-infected flowers by honeybees. In the honey, stored in the hive, *E. amylovora* is viable for less than a week. The persistence of *E. amylovora* at 4^{o}C in wax, bottom board debris, propolis and pollen is 3 weeks, 1 day, 1 day and 50 weeks respectively. At higher temperature the viability is much shorter. For example, in pollen stored at 35^o C, the in-hive temperature of the brood nest, the bacteria die within one week (Wael, 1988). On the honeybee the bacterium is viable for up to 2 days. Sabatini, et al. (2006) detected *E. amylovora* on honeybees and on bee-collected pollen in infected areas, prior to symptoms of fireblight were recorded

whereas in uninfected areas the bacterium could not be detected. Early detection of this plant pathogen in orchards can be an additional tool in the control process of this disease. These studies prove the feasibility of the honeybee colony to collect *E. amylovora* bacteria in detectable numbers on subsamples of bees and pollen in the fruit blooming period. Regular analysis of honeybees and/or bee products for the presence of *E. amylovora* during fruit flowering can be a tool to detect an *E. amylovora* flower infection in an early stage. For detection of *E. amylovora* during fruit flowering, subsampling of in-hive honeybees, hive-entering forager bees, pollen and honey are potentially usable. Honeybee colonies, placed in the proximity of flowering fruit orchards will both collect pollen and nectar from the orchards as from various other sources (paragraph 1.4). To increase the chance of subsampling sufficient material for detection a significant number of bees or pollen must be taken. Trapping pollen for analysis was not applied as pollen is an essential food for the honeybee colony and massive prolonged diminishing pollen income will affect the colony's development and foraging behaviour negatively (paragraph 1.4). Also honey was not sampled as the ripening process of nectar into honey will take, depending on the honey flow, up to multiple days with the risk of exceeding the viable period *of E. amylovora* in honey. In-hive bees were not used as taking these subsamples would result in a regularly disturbance of the colony. The hive-entering forager bee was chosen for subsampling the honeybee colony. Hive-entering bees are pollen foragers, nectar foragers returning from various food sources and orientating young bees that are not involved in the food collection process (par. 1.4). Consequently, an unpredictable part of the foragers, the ones that visited fireblight diseased *Rosaceae* flowers, carry *E. amylovora* bacteria. To overcome this "dilution" of bees that foraged on *Rosaceae* with bees that foraged on other plants and orientating bees, a substantial number of hive-entering bees or in-hive bees must be subsampled to have a significant chance to detect *E. amylovora* (par. 1.5).

In terms of honeybee colony sampling in bio-indication studies, the foraging bee samples material from the flowers and taking samples from a honeybee colony for bio-indication is depicted as subsampling. Subsampling bees from a honeybee colony can be done both by sacrificial subsampling (killing bees) and by non-sacrificial subsampling (not killing the bee and not affecting the colony's development and performance). Sacrificial subsampling of bees comes at the expense of the performance and survival of the colony. Therefore, sacrificial subsampling has its restrictions concerning the number of bees that can be sampled safely. An alternative sampling method to overcome the restriction of the limited number of bees that can be sampled is non-sacrificial subsampling.

In Austria at the Agentur Gesundheit Ernährungssicherheit (AGES), dr R. Moosbeckhofer developed a non-sacrificial subsampler. This non-sacrificial subsampler is based on adherence properties of the transparent plastic sheet (Caledonia NVG25) for *E. amylovora.* Bees walk over the Caledonia sheet and part of the bacteria will, if present, adhere to the sheet. In three successively bio-indication studies in 2012, 2013, AGES applied the non-sacrificial subsampling with the Caledonia sheets in Austria and Switzerland. *E. amylovora* could be detected on the Caledonia sheet prior to and simultaneously with visible symptoms of fireblight in the orchard (Halbwirth et al., 2014).
The non-sacrificial subsampler "Beehold device" has been developed at Plant Research International. The "Beehold device" is a non-sacrificial subsampler of honeybee colonies in which hive-entering and hive-leaving bees are forced to leave and enter the hive via different narrow tubes. The tube via which bees enter the hive (Beehold tube) is internally covered by a thin transparent PVC foliar holding a polyethylene glycol (PEG) layer and covered with gauze. The PEG layer adheres part of the particles attached to the hive-entering bee's hair and feet. The minimal adsorption rate of matter from the bee to the Beehold tube is set on 1%. Details of the application of the Beehold device are presented in chapter 5.
In 2013 the Beehold device and the Caledonia NVG25 sheet method were applied simultaneously in 2013 in a bio-indication study for *E. amylovora* in three orchards in Steiermark, Austria. The Beehold tube accumulates pollen are records the number of hive-entering bees.

6.2 Materials and Methods

The bio-indication scheme is presented in the flow chart on page 106 (Figure I).

6.2.1 Test orchards and non-sacrificial sampling

In April 2013, in orchards in Graz/Haidegg (GPS coordinates latitude, longitude 47.079415, 15.499346), Puch/Weiz (GPS coordinated 47.224767, 15.725748) and Nitscha/Gleisdorf (GPS coordinates 47.131449, 15.729584), at each location three normal functioning colonies, placed in the orchards for pollination, were selected. In the entrance of these hives, Beehold devices were inserted. Per hive one tube for hive-leaving bees and one Beehold tube for hive-entering bees was applied (Chapter 5).
The sampling scheme and duration of exposure are presented in Table 1. On the three apiaries subsampling with the Beehold device of the honeybee colony was done identically. The Beehold tubes for hive-

entering bees were replaced by new in-tubes at the end of each exposure period of 1, 2 and 5-days. From the start on the 29th April till 2nd May replacement was done by the author. From 3th May until 10th May the Beehold tubes were replaced by the beekeepers following the author's instructions and sampling scheme. The Beehold tubes were immediately after removal stored at 5° C. At the end of the entire exposure period, the Beehold tubes were collected by AGES and analysed at the AGES laboratories in Vienna. The tubes were processed till analysis according to paragraph 5.4.2. The PEG from each Beehold tube after exposure was dissolved in 1.5 ml phosphate buffer. From the resulting 3 ml PEG / phosphate buffer 1 µl was analysed with the quantitative polymerase chain reaction (qPCR). The qPCR amplified and quantified simultaneously the target DNA molecule, a partly sequence of the hypothetical protein AMY1267 of *E. amylovora* strain Ea273. The downstream primer hpEaF (5'CCGTGGAGACCATCTTTTA-3') and upstream primer hpEaR (5'AAGTTTCTCCGCCC-TACGAT-3') and FAM Taqman minor-groove binder hpEaP (5'TCGTCGAATGCTGCCTC-TCT-3') were applied. For the assays a Taqman Universal PCR Master Mix was used. The reactions were run in 20 µl volume using 0.5 mmol L^{-1} primers and 0.005 mmol l^{-1} probe and 1 µl template. The limit of detection (LOD) per reaction is 2 cfu *E. amylovora* (Gottsberger, 2010).

Table 1. Sampling scheme / duration of exposure of the Beehold tubes to the in-coming bees.

Exposure period		
1 day	**2 days**	**5 days**
29 April 2013	29 April 2013 to 30 April 2013	29 April to 03 May 2013
30 April 2013		
01 May 2013	01 May 2013 to 02 May 2013	
02 May 2013		
03 May 2013	03 May 2013 to 04 May 2013	
04 May 2013		04 May to 08 May 2013
05 May 2013	05 May 2013 to 06 May 2013	
06 May 2013		
07 May 2013	07 May 2013 to 08 May 2013	
08 May 2013		
09 May 2013	09 May 2013 to 10 May 2013	
10 May 2013		

At the same apiaries three colonies were provided with the AGES Caledonia non-sacrificial subsampler. Per hive, two PVC plastic tubes (diameter 32 mm, length 80 mm), inside covered by the plastic Caledonia

sheet (100 x 65 mm) are inserted in the flight entrance of the hive. The rest of the entrance is sealed with hard foam material, forcing both hive–leaving and hive-entering bees to pass the tubes. In these studies the tubes with the Caledonia sheets were replaced daily by the farmers on site and stored in the refrigerator till analysis. In the AGES laboratory the Caledonia sheets were rinsed off with phosphate buffer. The rinsing fluid was centrifuged and the sediment was resuspended in 200 ml phosphate buffer of which 1 µl was analysed according to the qPCR Gottsberger protocol as described above.

6.2.2 Minimal detectable number of sampled *Erwinia amylovora*

The 1.5 ml PEG from the Beehold tube was dissolved in 1.5 ml buffer, assuming no PEG was lost during the sampling process, resulting in 3 ml (3000 µl) PEG/buffer mixture. The mixture was homogenized and 1 µl of the mixture was analysed. To calculate the mean minimal number of *E. amylovora* bacteria per bee per day adsorbed from the bee's body to be detectable, the Beehold formula is applied (Chapter 5). In this formula the terms are 1) minimal detectable amount (LOD analysis protocol); 2) number of bees passed the Beeholdtube per day; 3) the minimal theoretic adsorption rate of matter from the bee's body to the PEG; 4) the assumed percentage of particles left on the bee's body after auto-grooming during foraging and the return flight plus part of the pollen in the corbicula and 5) the percentage of the bees that foraged on the target crop.

$$SCR\ \min = \frac{LOD}{n} * \frac{1}{F\ \min * F\ left}$$

Beehold formula

Herein are:

1. SCR min = specific minimal collection ratio, i.e. Minimal number of *Erwinia amylovora* bacteria a bee should collect per day to accumulate a detectable amount γ-HCH in the Beehold tube (number.day^{-1});
2. LOD, minimal detectable amount (LOD analysis protocol); (number)
3. n, number of bees passing the Beehold tube per one day (n.period^{-1});
4. F min, the minimal theoretic adsorption rate (fraction) of matter from the bee's body to the PEG in the Beehold tube = 0.01 (paragraph 5.4.2);
5. F left, the assumed fraction of particles left on the bee's body after auto-grooming during foraging and the return flight = 0.02 (paragraph 5.4.2).

6. In case the pollen show a certain fraction of the flowers available is visited. This fraction is taken into account by multiplying the SCR min by 1/ fraction target flowers visited.

6.2.3 Botanical origin of pollen

To estimate the percentage bees that foraged in the orchards during the exposure period, the percentage *Rosaceae* pollen has been determined in the PEG/buffer mixture. About 10 µl was placed on a microscope slight, quickly dried at 70° C on a temperature controlled heater and subsequently covered with fuchsin stained gelatin/glycerine (Kaiser) and finished with a cover glass. The determination of the pollen was done microscopically. Per slide, of hundred pollen grains the number of *Rosaceae* pollen and "rest" were determined.

6.3 Results

6.3.1 *Erwinia amylovora* on the PEG

On none of the dates, detectable amounts of *E. amylovora* were found. The results of the qPCR of the Beehold tubes are presented in Table 2.

Table 2. Results qPCR of *Erwinia amylovora* adsorbed to the Beehold tubes. Each Beehold tube, representing a specified exposure period was analysed apart. The number between brackets represents the number of Beehold tubes tested.

location	exposure		
	1 day[a]	2 days[b]	5 days[c]
Graz	< LOD (8*)	< LOD (6)	< LOD (2)
Puch/Weiz	< LOD (12)	< LOD (6)	< LOD (2)
Nitscha/Gleisdorf	< LOD (12)	< LOD (6)	< LOD (2)

* In Graz the observations stopped on 5 May 2013 resulting in less observations of the 1-day and 2-days exposure.

6.3.2 Number of hive-entering bees per day

The number of bees passing the Beehold tubes was recorded in one colony in Graz and one colony in Puch/Weiz. These colonies are assumed to be representative for the two other test colonies per apiary and the apiary in Gleisdorf. The data are presented in figure II.

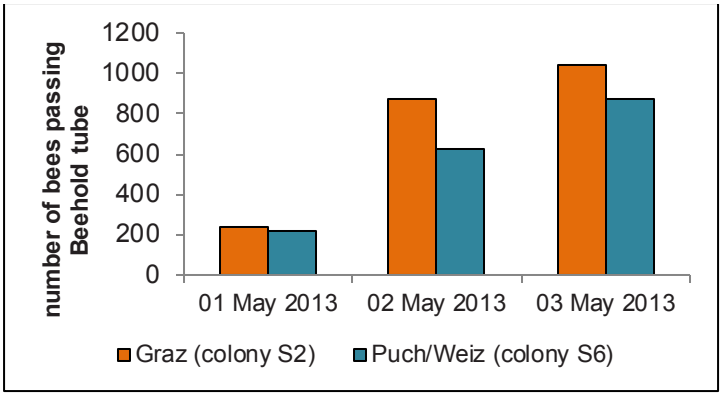

Figure II. Number of bees daily passing the Beehold tube in Graz and Puch/Weiz

6.3.3 *Rosaceae* pollen in Beehold tube

The percentages of *Rosaceae* pollen in the Beehold samples are presented in figure III.

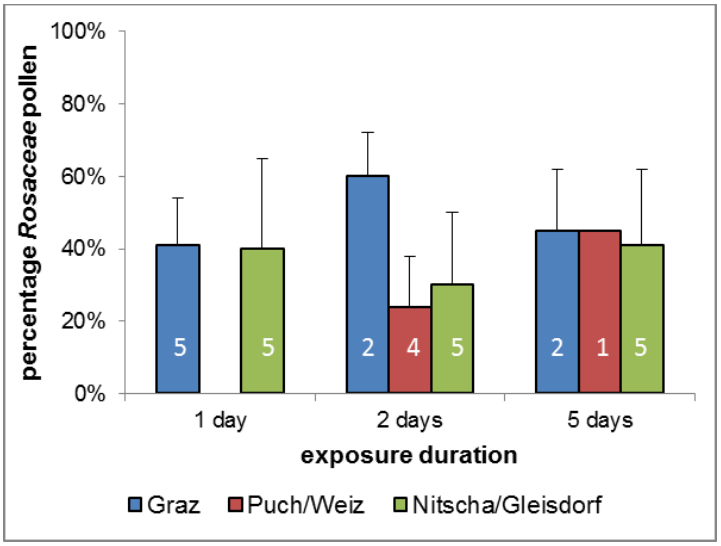

Figure III. Average percentage *Rosaceae* pollen in Beehold tubes containing > 100 pollen grains per Beehold tube. In the Beehold tubes of the 1 day exposure in Puch/Weiz, the percentage pollen did not exceed 100 grains.
The error bars on the columns represent the standard deviation. (sd). As the number of observation of the 5 day exposure at Puch/Weiz is 1, there is no sd.
For the Beehold formula the percentage bees foraging on *Rosaceae* is set on 40%.

6.3.4 The Beehold formula

The data of the terms and the result of the Beehold formula are presented in Table 3. In the first column the terms are presented. The 2 cfu in the second column is the Limit of Detection (LOD) of the analysis protocol. In the third column the result of the terms is presented: 6000 is the number of cfu's *E. amylovora* that must be present in the Beehold tube to have at least 2 cfu in a 1 µl sample for the molecular detection; the 1.5 ml PEG in the Beehold tube is dissolved in 1.5 ml buffer resulting in 3 ml (3000 µl) PEG buffer solution, to meet the 2 cfu per analysis 2 x 3000 = 6000 cfu's must be present. The average number bees is the result of counting the number of hive-entering bees passing the Beehold tube. The minimal adsorption of particles from the bee's body to the PEG is set on 1%. Of all the particles, pollen and other particles, about 2% remains on the bee's body after auto grooming and packing the pollen in the corbicula. As not all bees foraged on the *Rosaceae*, the percentage of Rosaceae pollen in the Beehold tube is considered to be representative for the percentage bees both nectar- and pollen foragers that foraged on the fruit flowers. The Beehold formula result is 1.15E+5, showing that each bee must collects minimally 1.15E+5 cfu *E. amylovora* to have a detectable number of the bacterium with the non-sacrificial Beehold sampling method.

Table 3. Terms and result of the Beehold formula

Term	LOD	Result term	Result Beehold formula
Minimal amount of cfu *E. amylovora* per in-tube	2 cfu	6000	1.15E+5
Average Number of entering bees.day^{-1}		650	
Minimal adsorption rate PEG		1 %	
Minimal percentage particles left on the bees body after [illegible]		2%	
Percentage hive-entering bees carrying *Rosaceae* pollen		40%	

6.4 Discussion

6.4.1 *Erwinia amylovora* in the Beehold device

No *E. amylovora* has been detected in the Beehold tubes. This result shows that the number of *E. amylovora* bacteria per Beehold tube was <6000 cfu *E. amylovora.* The outcome of the Beehold formula indicated that on average < 1.15E+05 cfu *E. amylovora* were present on each hive-entering bee after foraging. In Table 4, results of calculations are presented to estimate the number of cfu *E. amylovora* a bee must collect per trip given 650 hive-entering bees per day and 40% of the hive

entering bees visited *Rosaceae* flowers and the analysis of the PEG/buffer solution. The number of cfu *E. amylovora* in infected fruit flowers is 1E+5 to 1E+6 per flower (Pusey, 2002; Johnson et al., 1993).

Table 4. Estimation of number of infected flowers to be visited to collect a detectable number of cfu *E. amylovora*

term	data	result
Cfu / bee (result Beehold formula)	1.15E+5	
Estimated # cfu/flower	1E+5 to 1E+6	
# flowers to be visited per bee and all *E. amylovora* picked up in case the number of cfu *E. amylovora* per flower *is*	1E+5 cfu/flower	1.15 1.15
# flowers to be visited per bee and all *E. amylovora* picked up in case the number of cfu *E. amylovora* per flower is	1E+6 cfu/flower	0.12 0.12

The estimations indicate that, if there was a fireblight infection, *E. amylovora* could be detected in case each bee visited at least 1.15 or 0.12 infected flowers and had collected all *E. amylovora* bacteria present at a flower infection of respectively 1E+5 or 1E+6 cfu per flower were present. Concentrating the *E. amylovora* by centrifugation increases the chance to detect *E. amylovora* significantly. Possibly not centrifuging the PEG/phosphate buffer to concentrate particles has resulted in non-detectable numbers of *E. amylovora*. Adding a centrifugation step in the preparation for analysis of the PEG layer protocol will increase the chance of detecting *E. amylovora*.
The study of R. Moosbeckhofer (in Halbwirth et al., 2014) performed simultaneously with the Beehold testing, did not record *E. amylovora* as well in the Caledonia NVG25 sheets. These results are in line with the 2013 fireblight situation in the test orchards: no fireblight was observed in the orchards despite favourable conditions for an outbreak.

6.4.2 Fireblight in the test period

The Maryblyt[tm] prognosis model is applied in Austria to predict the chances for a blossom fireblight outbreak. It is based on 1) flowers open and stigma and petals intact; 2) accumulation of degree hours (DH) and accumulation of degree days (DD); 3) occurrence of dew and/or rain and 4) average temperature (Steiner, 1990). In Table 5 the results of the Maryblyt[tm] prognoses at the test orchards is presented.

Table 5. Maryblyt risks in the test orchards in 2013 (in Halbwirth et al.)

Date	Orchard		
	Graz	Puch / Weiz	Nitscha / Gleisdorf
29 April 2013	HT-	I	HW-
30 April 2013	HW-	I	HW-
1 May 2013	HW-	I	HW-
2 May 2013	I	I	I
3 May 2013	I	I	I
4 May 2013	M	M	M
5 May 2013	M	M	M
6 may 2013	M	M	M
7 May 2013	M	H	M
8 May 2013	H	H	H
9 May 2013	H	H	H
10 May 2013	H	H	H

Legend risks: L=low risk (1 condition present); M moderate risk (2 conditions present); H = high risk (3 conditions present; I = all 4 conditions for infection present; HW- = high risk but mean moisture term not met; HT- = high risk but mean temperature not met.

Although in the three test orchards during the test period the risk of fireblight was moderate to very high, no fireblight outbreak was recorded.

6.4.3 Number of foragers

The number of foragers counted at two test orchards in one colony per apiary, showed a limited number of foraging bees during day as recorded on day 3, 4 and 5 after the start of the study. This is less than expected 8000 to 10000 based on the colony size of approximately minimally 10 000 bees. The rule of the thumb is number entering bees = number of bees in the colony. This is partly due to the climatic circumstance that the bee season started very late in 2013, just before fruit bloom and partly due to ongoing learning process of the bees to enter the tubes.

6.4.4 *Rosaceae* pollen in the Beehold tube

The percentage pollen from *Rosaceae*, the host plant of *E. amylovora*, determined in the Beehold tube was in Graz, Puch/Weiz and Gleisdorf respectively 49%, 35% and 37% showing that the bees did not only forage in the orchards. This is in line with the phenomenon that bees focus their foraging on a limited number of main nectar and pollen sources (paragraph 1.4). For the calculation in the Beehold formula a 40% visit of foragers on *Rosaceae* is used. This percentage is based only on pollen, for nectar the same percentage is assumed.

Acknowledgement
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Chapter 7

Bio-indication with the non-sacrificial subsampler "Beehold tube" of atmospheric deposition of γ-HCH in the Bitterfeld region (Germany) and plants honeybees foraged on

J.J.M. van der Steen, J.T.C. Grotenhuis, H.H.M. Rijnaarts.

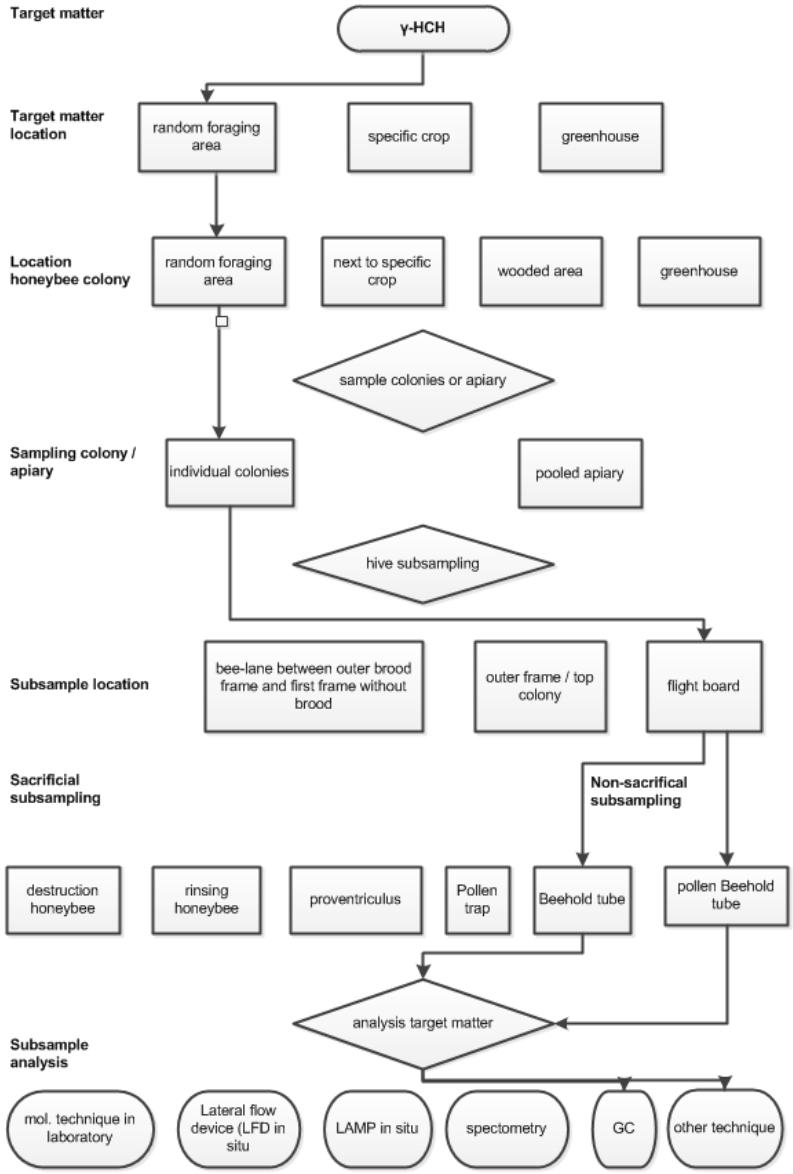


Figure I. Bio-indication flow chart: bio-indication with the non-sacrificial subsampler "Beeholdtube" of atmospheric deposition of γ-HCH in the Bitterfeld region (Germany) and plants honeybees foraged on

Abstract
In the Bitterfeld region, γ-HCH is one of the persistent organic pollutants (POP's) abundantly present in landfill sites and mining pits. This resulted in polluted soil, surface water, groundwater, river streambeds and sediments deposited on flood plains. Water transport mediated and atmospheric deposition of soil particles containing γ-HCH may pose a risk to the environment. In this study we investigated the application of the honeybee colony for bio-sampling of γ-HCH in the Bitterfeld region. The hypothetic route from streambed to the honeybee is: streambed + floodplain erosion ➔ wind erosion ➔ atmospheric deposition ➔ deposition HCH on flowers ➔ Honeybee colony sampling ➔ collecting HCH from honeybees by Beehold device ➔ analytical analysis of Beehold tube. We considered flowers as a qualitative representative receptor for areas of deposition of contaminants in the environment. Honeybees visit flowers to collect pollen, nectar, honeydew and water. In this process other non-floral matter such as atmospherically deposited particles, are collected simultaneously and unintentionally. We hypothesised that a detectable amount γ-HCH could be collected by a honeybee colony. For such a bio-indication, each foraging honeybee acts like a micro-sampler of the environment and the honeybee colony functions as the collector of these contaminants by transferring the contaminants to the Beehold tube. At three study sites in the Bitterfeld region the honeybee colonies were non-sacrificially subsampled applying the Beehold tube. The hive-entering bees were counted and the botanic origins of pollen from flowers bees have foraged on were recorded. The study period was June, July and August 2013. On average 8526 bees passed each of the two entrances of the hive through the Beehold tubes daily. The pollen records demonstrated a normal foraging behaviour of the honeybees at the three test sites, and thereby the functioning of the Beehold tube as an adequate sampler. Despite this, the relations between the presence of γ-HCH contaminated soil in the region and detection by the honeybee colony could not be made. The limit of detection of γ-HCH in the applied SPME / GC / EC analysis protocol is 0.4 µg γ-HCH. The passive load of particles in the hairy fur of bees is about 2% of the particles collected. Of those particles 1% is transferred from the bee's exterior to the Beehold tube. Based on this, each bee must collect minimally 235 ng γ-HCH per day to be detectable. No γ-HCH was detected in the Beehold tubes, indicating the γ-HCH contaminated fraction was < 0.001 ‰ of the matter collected by the bees.

7.1 Introduction

The Bitterfeld - Wolfen region, situated in Saxony-Anhalt in the Eastern part of Germany is known for two opposite features: *beauty* in the Bitterfeld Path (Bitterfeld Weg), the *ugliness* as a largest polluted megasite in Europe between 1930 – 2005, and again the *beauty* of a restored lake district landscape after the year 2005. The Bitterfeld Path was the amateur art movement (art and equality), announced in 1959 and 1964 by the former GDR (DDR) government in its struggle against revisionism, the tendency to favour reform above revolutionary change (Bazin, 2011). On the other hand, till the beginning of the 21th Century this region was known as an ecological disaster due its open lignite mining and chemical industry. Its industrial history dates back to half way the 19th Century (Bitterfeld Wolfen FAD).

From 1951 till 1982, in Bitterfeld - Wolfen, the pesticide Lindane (γ-HCH) was produced by Chemiekombinat Bitterfeld. Waste isomers from Lindane production (α, β, δ and ε-HCH) were dumped near the production sites on piles covered with soil and in empty open mine pits. Dumping sites of chemical waste including HCH and disused factories are pollution sources (Manz et al., 2001; Wycisk et al., 2013). After the reunification of Germany, the chemical industry was redeveloped and the landscape was restored by implementing a large nature redevelopment plan including green landscapes, wetlands and lakes. Old open mines were filled and others were transformed to lakes e.g. Grosser Goitzschesee. The megasite Bitterfeld was characterized by a regional pollution of soil, groundwater and surface water. Several studies have been conducted to describe the pollution of soil, groundwater and surface water (Briand et al., 2002; Kalbitz et al., 1997; Popp et al., 2000; Schwartz et al., 2006). Due to the extent of former dumpings of industrial chemical waste, chemicals could not be completely technically and economically removed. Thus the underground remained in certain regions heavily polluted, and an extensive set of measures were taken to reduce risks of spreading of the pollutants into the wider environment. The European Union funded WELCOME project, and the German federal government funded SAFIRA contributed strongly to develop this risk based contaminated megasite management approach (Wycisk et al., 2003; Wycisk et al., 2009). The result at present is a restored landscape, with pollution including HCHs still present in the region, and management measures to reduce risks to the environment. In this study, we apply a new method Beehold to test the environmental situation with respect to HCH contamination exposures.

HCH in topsoil

The soil in the Bitterfeld region is contaminated with γ-HCH and other isomers. The Ap-horizon which is the zone with dark, mineral and decomposed organic matter containing ploughed topsoil in the vicinity of emission sites was characterised. Total HCH content ranged from 5.22 to 11.5 $\mu g.kg^{-1}$ dry wt. and 5.25 to 10.0 $\mu g.kg^{-1}$ dry wt. at distances from the emitting source of 0 to 2.5 km and 2.5 to 10 km respectively. The γ-HCH concentrations near the emitting source ranged from 2.37 to 5.60 $\mu g.kg^{-1}$ dry wt. and further away from 1.54 to 5.23 $\mu g.kg^{-1}$ dry wt., an insignificant change with distance in this area investigated. In Ap horizons, Lindane (γ-HCH) can be completely decomposed or bio transformed in three years. Thus soils where γ-HCH predominates are suspected to receive new contamination inputs (Manz et al., 2001). In the Spittelwasser region (between the villages Wolfen and Jessnitz) the top soil contamination is 23.3 µg γ-HCH.kg^{-1} top soil (Schwartz et al., 2006). In the Spittelwasser region the HCH contamination of the soil spreads over about 40 km^2 (Wycisk et al., 2013). In the Netherlands the intervention value of γ-HCH in soil is 2100 $\mu g.kg^{-1}$ dry wt. (Rijkswaterstaat, 2013) In this perspective the values in the Bitterfeld region are rather low.

HCH in groundwater

Wycisk et al. (2003) reported HCH in groundwater and described the transport of contaminants to surface water of the river Mulde. The groundwater flows through the pollution sources and discharges partially into the river Mulde. The path of pollutants from groundwater through the streambed sediment into the surface water was described by Schmidt et al. (2008). After the flooding events in 2001, increased HCH concentrations have been detected in fish in the rivers Mulde and Elbe, showing remobilization of persistent organic pollutants including HCH, from polluted soils, sediments and deposits (Wycisk et al., 2013). Thus frequently flooded floodplains of the rivers Mulde and Elbe and in areas only flooded at high discharge situations, residues of HCH can be expected to be deposited, resulting in HCH polluted streambed and flood plains downstream of the polluted areas.

Passive sampling monitorings of HCH's

Several passive samplers have been developed and tested to monitor organic water- and soil pollutants. Passive sampling methods generally do not aim for quantitatively extraction and determination of the contaminant. Allan et al. (2006) presents passive sampling as a potential technology for water monitoring across Europe. Wennrich et al. (2003) recorded organic pollutants in the aquatic environment applying an integrative passive sampler consisting of a solid polydimethylsiloxane

(PDMS) rod or tube as sorbent of hydrophobic organic matter in an air or water filled low density polyethylene (LDPE) membrane tubing. Passive samplers of PDMS and polyoxymethylene (POM) can be applied to detect polycyclic aromatic hydrocarbons (PAH) in streambed sediment (Barthe et al., 2008). POM strips and PDMS tubing gave different results as PDMS overestimated and POM underestimated the availability of PAH in sediment. Head space solid-phase micro extraction (SPME) followed by gas-chromatography-electron capture detection to analyse soil samples for organic pollutants was described by Zhao et al. (2006). The transfer of pesticides through the atmosphere during and after application was studied by Briand et al. (2002) applying adsorption on a porous polymer (Tenax TA) followed by automatic thermal desorption and GC / MS analysis. These authors demonstrated that concentrations in the drift of the pesticide plume decreased downgradient with height, indicating a deposition of the compound into water and soils at the land surface.

HCH monitorings by Beehold

Honeybees visit flowers to collect pollen, nectar, honeydew and water. In this process unintentionally, other particles in the flowers such as atmospherically deposited particles are collected simultaneously. Each foraging honeybee acts like a micro-sampler of the environment and the honeybee colony as a passive sampler. As collected matter is in / on the honeybee, the honeybee colony is subsampled. Subsampling a honeybee colony can be done sacrificially; meaning bees are taken from the colony and destructed for analysis and non-sacrificially by collecting particles from the bee's exterior, without removing the bees from the colony. In this study the honeybee colonies were non-sacrificially subsampled with the Beehold device with the Beehold tube as the sampling part. Applying non-sacrificial subsampling is restricted to matter on the bee's exterior. Simultaneously the number of hive-entering bees and the botanic origin of pollen from flowers bees have foraged on were recorded with the bee counter in the Beehold device. For bio-indication study the honeybee colony is considered to be a passive sampling method (PSM).

Research question

Due to erosion of top soil- and catchment area of Spittelwasser, Mulde and Elbe streams and flood plain sediment in the Bitterfeld-Wolfen region, soil particles containing γ-HCH chemicals may have been spread via airborne transport into the regional environments followed by a deposition onto the flowers. When honeybees visit these plants and flowers, they can be assumed to collect a portion of these polluted particles. Based on the relative persistence of Lindane and other HCH's, the amount of HCH's still

present in the Bitterfeld-Wolfen region, potential spreading pathways from dump sites may occur. A likely pathway is that HCH's spread via groundwater to surface water to flood soil particles, that erode and are subsequently atmospherically mobilised and deposited as γ-HCH containing soil particles on plants and flowers by migration from underground sources via ground- and surface water to floodplain soil particles and a subsequent erosion and atmospheric spreading and deposition of HCH containing soil particles onto plants and flowers. This yields two research questions / hypothesises to be tested, and to be addressed in this study:

1. Is the HCH pollution present in the environment detectable via bio-sampling with the PSM honeybee colony? Through this research bio-indication of γ-HCH in flowers with non-sacrificial subsampling of the honeybee colony has been studied in the Bitterfeld-Wolfen region for the first time.
2. Is the above mentioned pathway of HCH spreading in regions such as Bitterfeld-Wolfen a factor that should be included into regional risk management strategies?

7.2 Materials and Methods

The bio-indication scheme is presented in the flow chart on page 120 (Figure I).

7.2.1 Study locations / position of apiaries

1. Wolfen, Germany (GPS coordinates latitude: 51.65566, longitude 12.26875). The apiary was located in the city centre of Wolfen directly behind the City Hall in the back yard of the apiculturist. The apiary was located approximately two km from the modern chemistry plants that have replaced the old Chemiekombinat facilities.
2. Muldestausee, OT Friedersdorf, Germany (GPS coordinates latitude: 51.65148, longitude 12.36197). The apiary was located in a rural area in the vicinity of the largest and water refilled mining pit (Grosse Goitzschesee) and the Muldestausee.
3. Muldestausee, OT Brösa, Germany (GPS coordinates latitude: 51.61242, longitude 12.51240). The apiary was located in a rural area near the forest, the abandoned test garden of the Chemistry Plant and the river Mulde.

At each apiary, three fully occupied (approximately 15,000 – 20,000 bees) were selected. Each colony was provided with the Beehold device. In the Beehold device, the Beehold tube is the sampling unit. The Beehold device is described in detail in Chapter 5. Per apiary one bee counter was

connected to the Beehold device. The number of hive-entering bees of one colony was assumed to be representative for the activity of the two other colonies. Per colony two Beehold tubes and two out-tubes were installed. Per colony one Beehold tube contained PEG 3 (mixture 50 % PEG 1000 + 50 % PEG 1500) and the other Beehold tube contained PEG 4 (mixture 25 % PEG 1000 + 75 % PEG 1500). To 1.5 ml PEG mixture, 5 mg C18 was added to improve adherence of lipophilic matter.

In Figure II, a single Beehold device with two Beehold tubes (left) and the three test colony in the Friedersdorf apiary are presented.

Figure II. Beehold devices in practise as installed at the apiary Wolfen. The hive was provided with two Beehold tubes and two out-let tubes (left)

Apiary Friedersdorf. Three hives provided with the Beehold device (right)

7.2.2 Lindane (γ–HCH)

The reference Lindane applied in the preliminary calibration studies was Lindane: 1α, 2α, 3β, 4α, 6β, hexachlorocyclohexane γ-HCH 97%. In this report referred to as γ-HCH (Sigma-Aldrich).

7.2.3 Beehold device

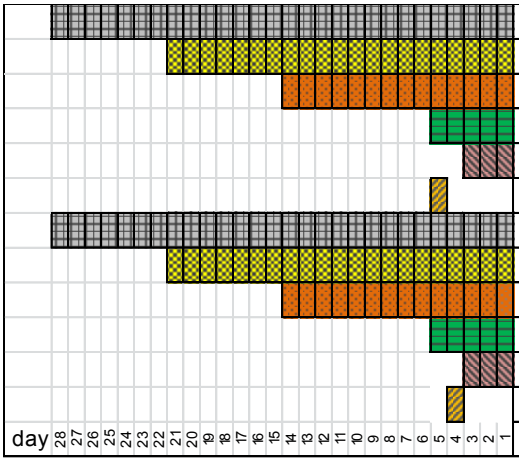
The Beehold device is a non-sacrificial subsampling device for honeybee colonies. The Beehold device splits the hive-leaving and hive-entering bees. The hive-entering bees pass the Beehold tube. The Beehold tube is internally covered with a thin transparent PVC layer covered with polyethylene glycol (PEG). PEG is a moderately sticky material and adsorbs about 1% of the particles from the bee's exterior. The Beehold device can be expanded with a bee counter to record the number of hive-entering bees. The Beehold device is presented and discussed in detail in Chapter 5.

7.2.4 Installation of the Beehold device

Each hive entrance was sealed with hard foam with 6 to 8 openings to determine the most frequently used entrance location per hive. After about one to two hours the hive-entering- and hive-leaving bees were used to the new openings. Beehold devices with empty Beehold tubes were placed in the two most frequently used entrance holes and the out-tubes in two other holes. The remaining openings were sealed. Again after about one hour the bees were used to the Beehold tubes and the Beehold tubes containing PEG were inserted.

7.2.5 Exposure and non-sacrificial sampling

At each apiary, hives 1, 2 and 3 were provided with two Beehold tubes. During the entire study period the same colonies were used. Exposure was studied in four periods. In the first exposure period ranging from 23 – 27 June 2013, colony 1, 2 and 3 were provided with Beehold tubes for 1, 3 and 5 days respectively. In the second exposure period ranging from 28 June to 25 July, the three colonies were provided with Beehold tubes for 14, 21 and 28 days respectively. The same scheme was repeated in the third exposure period ranging from 26 – 30 July and the fourth exposure period ranging from 31 July to 28 August 2013. In the first and third exposure period, replacement of the Beehold tubes was done by the corresponding author. In the second and fourth period replacement (taking out the used tubes and inserting a new Beehold tube) was done by the beekeeper according to the author's instruction. After exposure the Beehold tubes were stored at 5° C. for a maximum of four weeks and then at -20° C, until analyses. During the periods when no Beehold tubes were provided, the Beehold tubes were replaced by empty tubes. At the three apiaries the sampling scheme was applied simultaneously with a delay between the apiaries of about 30 minutes, starting in Wolfen, then Friedersdorf and last Brösa. Sampling was done according to Figure III.



hive id	Beehold tube in	Beehoild tube out
hive 3	31 July 2013	27 August 2013
hive 2	31 July 2013	20 August 2013
hive 1	31 July 2013	13 August 2013
hive 3	26 July 2013	30 July 2013
hive 2	26 July 2013	28 July 2013
hive 1	26 July 2013	26 July 2013
hive 3	28 July 2013	25 July 2013
hive 2	28 July 2013	18 July 2013
hive 1	28 June 2013	11 July 2013
hive 3	23 June 2013	27 June 2013
hive 2	23 June 2013	25 June 2013
hive 1	26 June 2013	26 June 2013

Figure III. Sampling / exposure scheme. Each coloured / striped box indicates the period hive-entering bees passed the Beehold tube. The first column represents the day start from 1 till 28 days. For example, in the second row from below (26 June 2013 till 26 June 2013) the hive entering bees of hive 1 passed the Beehold tube for 1 day. In the fourth row from below (23 June till 27 June) the hive-entering bees of hive 3 passed the Beehold tube for five days. In the third row from above (31 July 2013 till 13 August 2013), the hive entering bees of colony 1 passed the Beehold tube for 14 days.

7.2.5 Analysis of Beehold tubes

Sampling resulted in 2 X 12 Beehold tubes per apiary. From each sampling period one Beehold tube (PEG 3) was analysed for γ-HCH. The second Beehold tube (PEG 4) was used for pollen analysis to determine the crops the bees foraged on.

Figure IV. Bluecap with rubber septum

γ-HCH on the PEG/C18 film was analysed with head space solid phase micro adsorption (SPME) followed by GC/EC analysis. The column applied in the GC was a Varian CP-Sil 24CG column (coating FS 30x25 (.25)).

To analyse γ-HCH adsorbed on the PEG layer, the Beehold tube with the PEG/C18 fil was placed in a 50 ml blue cap tube. The cap was mounted with a rubber septum for needle passage (Figure IV) to insert the SPME fibre. Next the adsorption SPME fibre (PDMS 100 µm) was inserted in the Beehold tube and left at room temperature for 15 minutes for adsorption. After the adsorption period, the SPME fibre was taken out and inserted into the 200

°C GS inlet. The SPME adsorption fibre remained in the inlet for a desorption period of 10 minutes. After removal of the SPME fibre the GS analysis was started according to a pre-set program: starting at 40 °C and increasing the temperature every minute with 12 °C. to 220 °C followed by a 5-minute period of 220 °C. The carrying gas was nitrogen. γ-HCH was detected with an ECD (electron capture detector) by recording the time based GC/ECD fixed at 0.01 second detections. Per analysis the runtime was 21 minutes. The γ-HCH peak was at approximately 12 minutes.

7.2.6 Determination of the limit of detection (LOD) of γ-HCH on the PEG / C18 layer

To determine the LOD according to the head space solid phase micro adsorption (SPME) followed by GC/ECD analysis applied, a concentration range was tested. Per PEG/C18 layer as applied in each Beehold tube, 40 mg Lindane / bentonite mixtures containing 0.4 µg, 0.8 µg, 1.2 µg, 1.6 µg, 2 µg, 4 µg, 6 µg, 8 µg, and 10 µg Lindane were analysed. 40 mg is the maximum amount of matter that adheres to the PEG at room temperature in a 24-hour period.

7.2.7 Calculations on the minimal amount γ-HCH a bee should collect per day to accumulate a detectable amount γ-HCH in the Beehold tube

To calculate the mean minimal amount of γ-HCH a bee must collect per day to accumulate a detectable amount in the Beehold tube, the Specific minimal Collection Ratio (SCR), the Beehold formula is applied (Chapter 5).

Beehold formula

$$SCR\ \min = \frac{LOD}{n} * \frac{1}{F\ \min\ * F\ left}$$

Herein are:

1. SCR min = specific minimal collection ratio, i.e. Minimal amount γ-HCH a bee should collect per day to accumulate a detectable amount γ-HCH in the Beehold tube (weight.day^{-1});
2. LOD, minimal detectable amount (LOD analysis protocol); (weight);
3. n, number of bees passing the Beehold tube per one day (n.period^{-1});
4. F min, the minimal theoretic adsorption rate (fraction) of matter from the bee's body to the PEG in the Beehold tube = 0.01 (paragraph 5.4.2);
5. F left, the assumed fraction of particles left on the bee's body after auto-grooming during foraging and the return flight = 0.02 (paragraph 5.4.2).

7.2.8 Limit of Detection LOD

The Limit of Detection of γ-HCH on PEG/C18 calibration mixture and analysed according the protocol for head space solid phase micro adsorption (SPME) followed by GC/ECD analysis is 0.4 µg γ-HCH. The result of the calibration is presented in the figure V (test range 0 – 10 µg), figure VI (0.05 – 0.4 µg) and figure VII (0.4 – 10 µg).

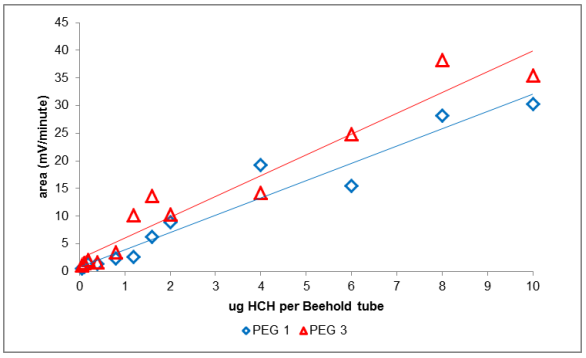


Figure V. Calibration line ranging from 0.05 µg till 10 µg γ- γ-HCH
PEG 1 y = 3.13x + 0.83, R^2 = 0.95
PEG 3 y = 3.77x + 2.26, R^2 = 0.94
LOQ = 2 ug HCH

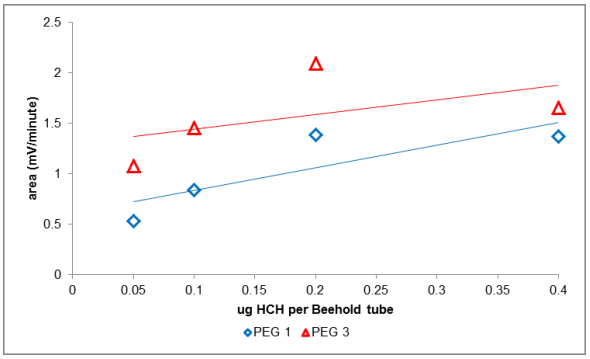


Figure VI. Calibration line ranging from 0.05 µg till 0.4 µg γ-HCH
PEG 1 y = 2.25x + 0.61, R^2 = 0,68
PEG 3 y = 1.43x + 1.30, R^2 = 0.28

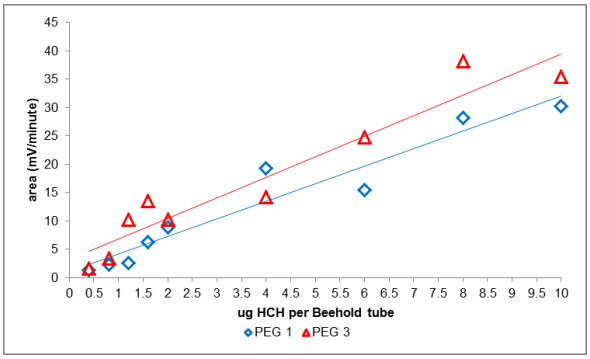


Figure VII. Calibration line ranging from 0.4 µg till 10 µg γ-HCH
PEG 1 y = 3.10x + 1.05, R^2 = 0.93
PEG 3 y = 3.63x + 3.15 R^2 = 0.92

7.2.9 Pollen analysis

To determine the flowers bees foraged on during the exposure period, pollen origin and quantity was identified. For this the PEG layer was dissolved in 1.5 ml tap water and subsequently 500 µl was pipetted in an Eppendorfer. In a centrifuging step (10 minutes at 14000 rpm) the pollen was concentrated. After centrifugation the supernatant was poured off and the remaining pellet was re-suspended in the approximately 40 µl supernatant. Next 10 µl of the pollen suspension was pipetted onto a microscope slide, dried at 70 °C on a temperature controlled heater, covered with fuchsine stained gelatin-glycerine (Kaiser's formulation), sealed with a microscope cover glass and stored at room temperature. The pollen was identified microscopically for the morphological characteristics using reference pictures, drawings and descriptions (Hodges, 1974; von der Ohe & von der Ohe, 2001).

7.3. Results

7.3.1 Number of hive-entering bees per Beehold tube

The number of hive-entering bees per Beehold tube is presented in Table 1. On average 8526 hive-entering bees passed the Beehold daily. Per colony, both Beehold tubes were used equally (visual checks) the number of hive-entering bees is 2 x the number of bees passing the Beehold tube with the bee counter.

Table 1. Average number of hive-entering bees via the Beehold tube and in the hive

Study period	Number of bees passing daily the Beehold tube (recording, sd)	Hive-entering bees per colony
23 – 27 June 2013	8181 (8, 3783)	16362
25 – 29 July 2013	8921 (7, 9012)	17842
Study mean	**8526 (15, 6489)**	**17051**

7.3.2 Minimal detectable amount γ-HCH per bee per day

The terms of the Beehold formula are:

- LOD analysis protocol: 0.4 µg
- n = average number of bees that passed the Beehold tube per day: 8526
- F min = fraction adhered to Beehold tube: 0.01
- F left = fraction particles left after self-grooming: 0.02

The outcome of the Beehold formula is that SCR min = 235 ng γ-HCH on average which is the amount of γ–HCH each bee must collect per day to give detectable γ–HCH readings of the Beehold sampler. The number of bees that passed the Beehold tube daily varied due to weather and colony conditions. The minimum and maximum number of bees that passed the Beehold tube daily was 2344 and 25750 respectively. The resulting SCR min = 853 ng in case 2344 bees passed the Beehold tube and 78 ng in case 25750 bees passed.

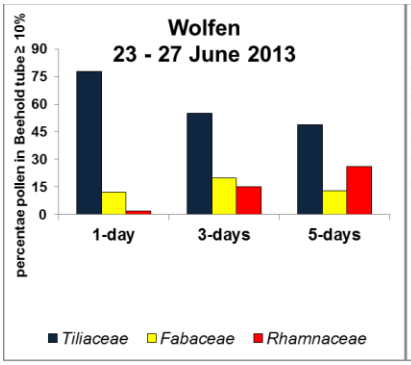
7.3.3 γ-HCH in the Beehold tubes, exposed to passing honeybees at the three study sites

In none of the 3 x 12 (study site x exposure period) analysed Beehold tubes γ-HCH was detected. Each analysed Beehold tube contained < 0.4 µg γ-HCH.

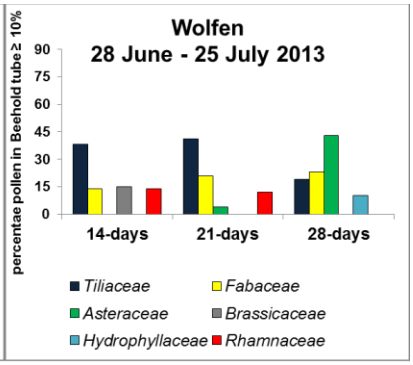
7.3.5 Pollen

Pollen in the Beehold tube show (1) where the bees foraged on and (2) the functionality of the Beehold tube. The botanic origin of the pollen is identified to Family level. For each Beehold tube 100 pollen grains were identified and the percentage per botanical origin was calculated. The data per exposure period: 23 – 27 June, 28 June – 25 July, 26 – 30 July, 31 July – 27 August and study site were pooled. Pollen of botanic families present in Beehold tube at least ≥ 10% are presented in Figures VIII, IX, X. These botanic families are considered to be major foraging sites for the honeybees. Pollen that was present < 10 is listed below the corresponding figures. These botanic families are considered to be minor foraging plants in that specific period. The data show on all sites and during the four study periods three to five major foraging sites and maximally 12 minor foraging sites. In Wolfen, *Tilia* spp. (lime tree) was a major foraging site in

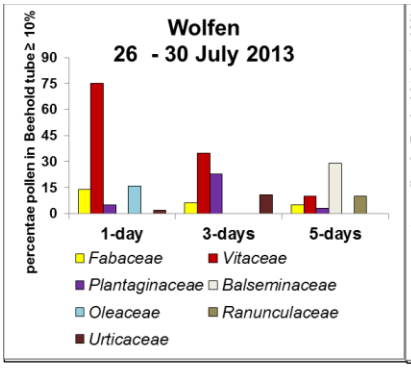
the June and July periods, *Fabaceae* spp. (e.g. clover) was a rather constant pollen and nectar yielding botanic family during the entire study period. In Friedersdorf in the beginning *Fabaceae* spp were important plants bees foraged on, later on this position was taken over by *Balsaminaceae* (Balsam). In Brösa *Fabaceae* was a constant nectar and honey yielding plant, followed by *Asteraceae* (e.g. dandelion, daisy and sunflower) and *Balsaminaceae*. At all sites the bees foraged on a variety of flowers, indicating they were exposed to, if present, atmospheric deposition of for example soil particles.



< 10%
Brassicaceae
Plantaginaceae
Oleaceae
Pinaceae
Poaceae (grass)
Rosaceae
Solanaceae
Urticaceae



< 10%
Ranunculaceae
Plantaginaceae
Vitaceae
Lamiaceae
Poaceae (grass)
Oleaceae
Solanaceae
Urticaceae
Papaveraceae



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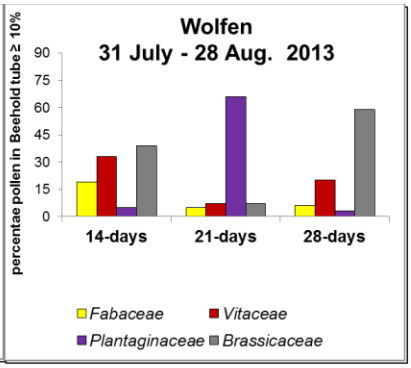
Tiliaceae
Caprifoliaceae
Curcubitaceae
lamiaceae
Oxidaceae
Pinaceae
Poaceae (grass)



< 10%
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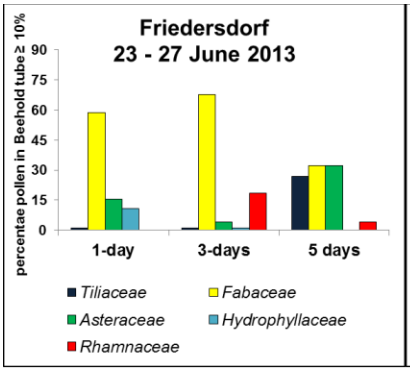
Apiaceae
Caprifoliaceae
Oleanaceae
malvaceae
Poaceae (grass)
Ranunculaceae
Solanaceae
Taxaceae
Urticaceae

Figure VIII. Pollen in the Beehold tubes in Wolfen



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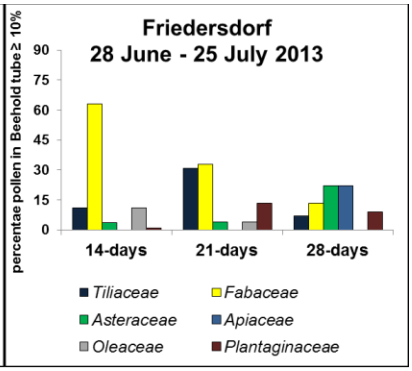
Caprifoliaceae
Oleaceae
Pinaceae
Rosaceae
Ranunculaceae



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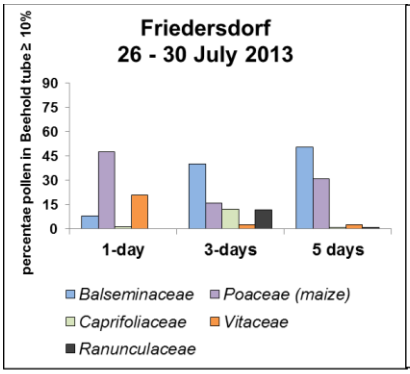
Hydrophylaceae
Liliaceae
Poaceae (grass)
Polygonaceae
Rosaceae
Ranunculaceae
Solanaceae
Urticulaceae
Vitaceae
Rhamnaceae



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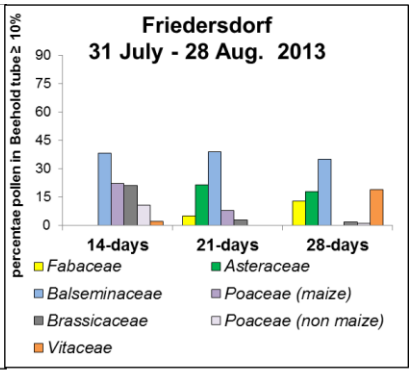
Oleaceae
Malvaceae
Rosaceae
Polygonaceae
Plantaginaceae
Urticaceae



< 10%
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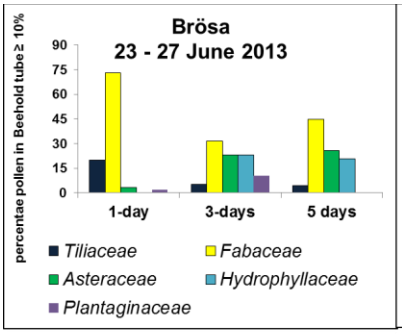
Caprifoliaceae
Chenopodiaceae
Dipsacaceae
Hydrophylaceae
Lamiaceae
Lythraceae
Oleaceae
Polygonaceae
Plantaginaceae
Rosaceae

Figure IX. Pollen in the Beehold tubes in Friedersdorf



< 10%
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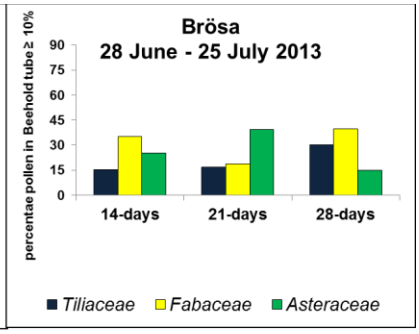
Caprifoliaceae
Chenopodiaceae
Solanaceae
Vitaceae



< 10%
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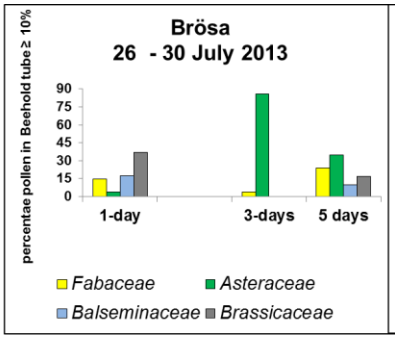
Hydrophylaceae
Caprifoliaceae
Lamiaceae
Poaceae (grass)
Plantaginaceae
Rosaceae
Solanaceae
Urticulaceae
Rhamnaceae



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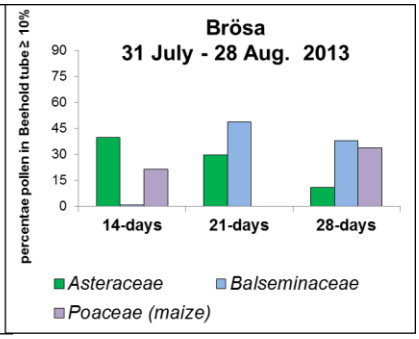
Poaceae (maize)
Caprifoliaceae
Caryophylaceae
Oleaceae
Polygonaceae
Plantaginaceae
Rosaceae
Solanaceae
Violceae
Vitaceae



< 10%
Fabaceae
Brassicaceae
Caprifoliaceae
Chenopodiaceae
malvaceae
Plantaginaceae
Vitaceae

Figure X. Pollen in the Beehold tubes in Brösa

7.4 Discussion

7.4.1 Foraging activity

The mean number of hive-entering bees is as expected, reflected by the colony size. In summer, the number of hive-entering bees per day is around the total number of bees in the colony. The pollen in the Beehold tubes show that the bees foraged on 3 – 5 main crops and up to 12 minor crops. This is a normal foraging pattern (paragraph 1.4). Based on colony size, the foraging activity and variety of visited crops, the honeybee colonies can be regarded as normal representative active colonies, suitable for bio-indication.

7.4.2 γ-HCH on the hive entering bee

The γ-HCH load on a hive-entering bee (before passing the Beehold tube) can be estimated beforehand by an assumption of different fractions γ-HCH (permilles) in particles (floral and non-floral) collected by honeybees and the average passive particle load after auto-grooming on the bee's exterior of 0.4 mg as described in paragraph 1.6.1. Fractions of 0.1‰, 0.01‰, 0.001%, 0.0001‰ and 0.00001‰ γ-HCH will result in a γ–HCH load of 40 ng, 4 ng, 0.4 ng, 0.04 ng and 0.004 ng. The longer the honeybee forages on contaminated food sources, the more γ-HCH will be collected (Table 1). The data presented in Table 2, are depicted as straight lines (A, B, C, D) in figure XI.

To accumulate a detectable amount γ-HCH in the Beehold tube during the studies exposure periods, the minimal mean amount each bee should collect daily is 235 ng. Due to the cumulative feature of the Beehold tube, the longer the period bees will pass the Beehold tubes (exposure period) the less γ-HCH each bee has to carry to accumulate a detectable amount in the Beehold tube. The required minimum amount γ–HCH.bee^{-1}.day^{-1} for the exposure days tested is shown in Table 2.

Table 2. The calculated γ-HCH in permilles of the 0.4 mg passive load of the hive-entering bee and the required amount γ-HCH.bee^{-1}. day^{-1} to be detectable

foraging days	amount (ng) γ-HCH.bee^{-1}.day^{-1} in passive load					minimal detectable amount (ng) γ-HCH.bee^{-1}. day^{-1}
	Fraction γ-HCH (‰) in passive load of particles					
	0.1	0.01	0.001	0.0001	0.00001	
1	40	4	0.4	0.04	0.004	235
3	120	12	1.2	0.12	0.012	78
5	200	20	2	0.2	0.020	47
14	560	56	5.6	0.56	0.056	17
21	840	84	8.4	0.84	0.084	11
28	1120	112	11.2	1.12	0.112	8

In Figure XI, the minimum required amount to be detectable are line-depicted in the function of time (ng γ–HCH.bee^{-1},day^{-1}). In figure XI it can be read that in case on average 0.1 ‰, 0.01 ‰, 0.001 ‰ of the particles collected contained γ-HCH, it would have taken about 3, 8, 24 days of accumulation in the Beehold tube to obtain sufficient γ-HCH for detection. Contamination of 0.0001 ‰ γ-HCH is not detectable within four weeks exposure and accumulation.

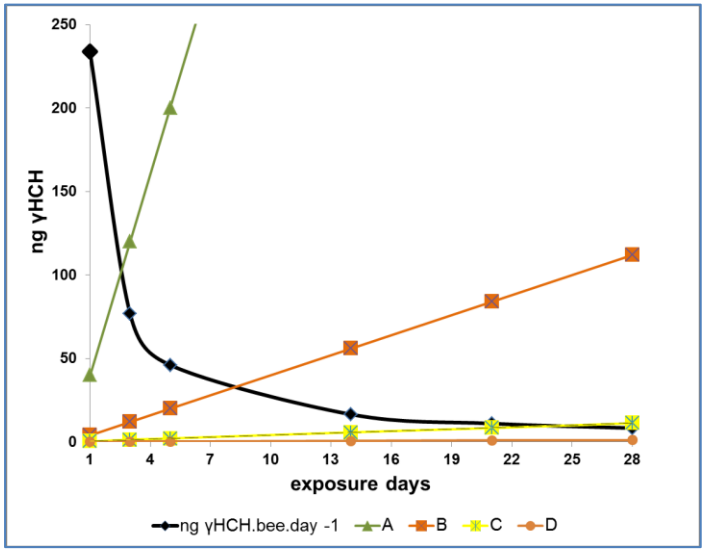


Figure XI. SCR min in ng γ-HCH.bee^{-1}.day^{-1} for achieving the detection limit.
A: 0.1 ‰ γ-HCH in passive particle load;
B: 0.01 ‰ γ-HCH in passive particle load;
C: 0.001 ‰ γ-HCH in passive particle load;
D: 0.0001 ‰ γ-HCH in passive particle load.

In the Spittelwasser region near study site Wolfen, surface soil particles contain 23.3 µg γ-HCH.kg^{-1} topsoil (Schwartz et al., 2006). Ergo, each mg topsoil contains $\left(\frac{23.2}{1000000}\right) * 1000) = 0.0233$ ng γ-HCH.mg^{-1}. Assuming each mg topsoil contains 0.0233 ng γ-HCH, this amount corresponds with a contamination of 0.0001‰ to 0.00001‰γ-HCH in the passive particle load of a honeybee to be brought in per day (Table 2). This amount is not detectable with the analytic method used and PSM honeybee colony by non-sacrificial sampling. Contamination should be a factor 100 higher to be detectable.

7.4.3 Sacrificial sampling versus non-sacrificial sampling

The mean number of bees passing the Beehold tube daily is 8526 corresponding with sacrificial sampling of 86 hive-entering bees per day. In the 28-days observation period about 2400 bees must be sampled to equalize non-sacrificial sampling. This would affect the performance in terms of number of foragers and food supply and development of the colony severely and would not have been an alternative for non-sacrificial sampling.

Working with honeybee colonies with more foragers, pooling (apiary) long exposed Beehold tubes and improving the adsorption capacity might increase the amount γ-HCH to a detectable level.

7.5 Conclusion

The route of γ-HCH from contaminated topsoil and contaminated streambed sediment via atmospheric deposition of soil eroded particles into flowers could not be established. The relation between contaminated soil and honeybees cannot be made in this case. However, the use of the pollen analysis as an internal standard showed the Beehold tube functions well as environmental bio-sampler.

Further research on the use of the honeybee colony for bio-sampling and detection of γ–HCH soil pollution is needed under more controlled conditions, with i.e. different levels of HCH contaminated soils and known deposition levels on plants and flowers.

The concept presented in Table 2 and depicted in Figure XI, incorporates the LOD of a certain component, the SCR min and the assumed fraction of contamination of particles in the passive particle load on the bee's exterior. This concept can be used as generic model to predict the honeybee colony can be used for an estimation of successful bio-indication with the PSM honeybee and applying sacrificial or non-sacrificial subsampling.

Acknowledgement

The author acknowledges Dr H. Kaatz for his great collegiate help to organize this study in Germany. My gratitude to mr M. Brandl, mr R. Mauser and Mr H. Kapke for the access to their apiaries, working with their honeybee colonies and their hospitality and cooperation. Thanks to Mr J. Kubiak and Mr H. Beijleveld for their great support and help in the laboratory. We thank Chula Hok-Ahin for her help in identifying the botanic origin of the pollen. This study was made possible by the financial support of the business unit Biointeractions and Plant Health of Plant Research International Wageningen UR.

Chapter 8

General Discussion

8.1 The honeybee colony as a bio-sampler

Although many studies have demonstrated that the honeybee colony is as suitable as many other passive sampling methods, the PSM honeybee colony has its restrictions. In my commentaries I will follow features of the honeybee colony as PSM (paragraph 1.3.2) and the source-path-receptor concept of target matter (paragraph 1.2).

8.1.1 Features of the honeybee colony as Passive Sampling method

Following the popular press, the honeybee colony is not the superorganism within the meaning of a biological superorganism but as an environmental superorganism, a gauge of the wellbeing of the environment. Indeed, the honeybee colony has its role in the biological hierarchy with a significant role as pollinating insect especially in horticulture and agriculture. However, it is not a bio-indicator super-organism. Like all other bio-indicators it has its biological restrictions. In general, the order of age related tasks, the many thousands of daily foraging trips, it's careful and complete food collection by removing pollen and nectar from the flower by scavenging the flowers, the accumulation of intentionally collected food and unintentional collection of matter present in flowers are significant positive aspects of the honeybee colony as a passive sampling method. On the other side, the limited active period in the moderate climate zones of about six months, its communication system directing large cohorts of forager bees to a limited number of profitable food sources within the foraging areas with the preference to collect the food as close to the hive as possible are restrictions as generally bio-indicators cover a larger area. On the other hand, these features are advantages as bio-indicator for specific plant pathogens.
Pollen foragers accumulate the collected particles during collection and the home-flight in the corbicula. Some of the collected particles remain present on the fury hairs and on the feet of the bee. At first sight this makes the pollen pellets in the corbicula the perfect object to sample and analyse. This is the case for pollen-bound plant pathogens and lipophilic pesticides. Frequent daily subsampling large amounts of pollen (ounces), will affects the colony negatively. Based on the annual food need of a colony there are about five times more nectar foragers than pollen foragers. The non-frequent auto-grooming by the nectar foragers leaves particles in the hair and on the feet (Westerkamp, 1991). This very presence of nectar foragers being less clean plus the pollen foragers being clean to a certain extent makes the forager cohort of the honeybee colony a suitable general scavenging insect for bio-indication.

Bees collect pollen, nectar and water in a restricted foraging area and limited number of different food sources taking into account the distance and energy profits. For atmospheric deposition of particles containing POP's or heavy metals it is not important; all flowers in the foraging area will be contaminated. However, in cases where the plant pathogen is on a plant less attractive than the target plant, part of foragers cohort will ignore this plant / food source. This is not complete as there are scout bees continuously looking for new food sources visiting many potential sources. Not only for the bio-indication of plant pathogens but also for pesticides this is an aspect to keep in mind.
In the Coloss project CSI pollen about 500 beekeepers in 21 countries counted in 3-week intervals the number of different coloured pollen. The 2014 results show a mean of seven colours independent to the land use, confirming the restriction of honeybees to a limited number of foraging sites (in this study pollen foraging sites).
In Figures I, II, III, the pictures (August 2015), provided by Mr J. van Popering, a Dutch participant of the CSI pollen project show the very diverse availability of flowering plants and the resulting limited number of colours of pollen, showing bees are selective in their foraging activity.

Figure I. Flowering plots in the 500 meters from the apiary

Figure II. Flowering plots in the 500 meters from the apiary

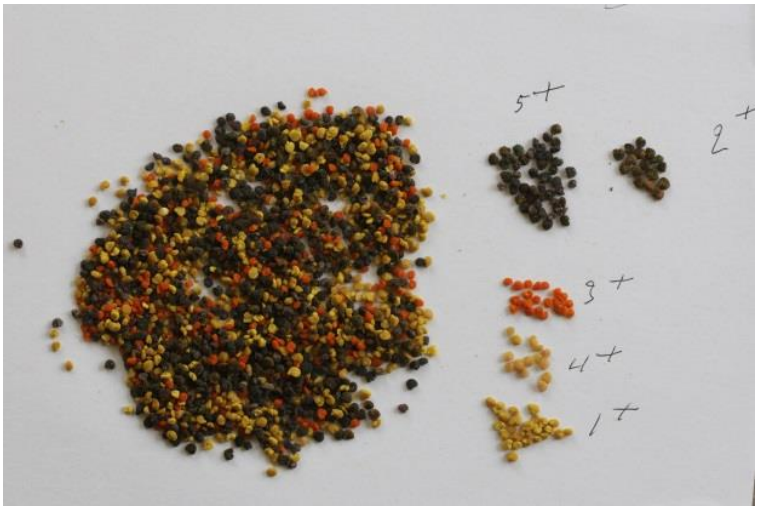


Figure III. Five different colours of pollen collected by the bees

Bees of an apiary divide themselves over the foraging area exploiting partly similar and partly different food sources. In case the target matter is in a defined site or on specific plants more than one colony within the apiary must be sampled to lower the risk of sampling a colony not foraging on the target plant.
In-hive exchange of particles is an important feature of the honeybee colony. Due to this behaviour target matter will be on all bees in the colony within hours. This makes every bee in the colony both hive-entering bees and in-hive bees a subsample object. It is obvious that there is a dilution- and temporal effect, small amounts of target matter entering a strong colony may result in undetectable amount of or no target matter on the in-hive bees. A non-continuously inflow of target matter will result in a decrease in the hive due to mortality of old bees and the emergence of new born bees. On the other hand, a constant influx of a contaminant or plant pathogen will increase the amount per in-hive bee.
The number of 5-6 million annual foraging trips, visiting multiple flowers per trip is an important advantage of the PSM honeybee colony no other PSM can complete with. It compensates partly the disadvantages mentioned above.

8.1.2 Source-Path-Receptor of POPs, heavy metals and plant pathogens

Persistent organic pollution (POP)
The bio-indication of persistent organic pollutions (POPs), from sites such as dumps and contaminated soils/groundwater, are not relevant for the foraging honeybee as the source is inaccessible to the bees. The path of these contaminants goes via surface water, streambed sediments and soil erosion to the recipient flowers. During the path, the honeybee may encounter this contaminant by drinking water from contaminated waters and by colliding during the foraging trips. Bio-indication of contaminated water by honeybees can be considered as a less relevant connection point. Bees collect their water from various sources such as plant guttation fluid, puddles and ponds. Inside the hive they collect condensation fluid produced during the honey ripening process. Collision with soil eroded POP containing soil particles might occur. However as shown in the study about the relationship between heavy metals in the air and in/on honeybees (Chapter 3), it is unlikely to detect POPs in this way. The order of magnitude must exceed the reference concentration in ambient air, one or two times to be detectable. This may occur locally but is unlikely over a

large area due to the plume form of contaminant path in the air. Atmospheric deposition of soil eroded streambed- and flood plain soil particles will deposit on flowers and be collected by the foraging bee. Depending on the amount of soil particle deposition and concentration of the POP in the soil particles this might be detectable. Assuming the amount and concentration are low, it takes many bees to collect a detectable amount (Chapter 7). For bio-indication, the accumulation is preferably in the non-sacrificial sampler as this device has no sample limit, while subsampling hive-entering bees and in-hive bees has its sample size limitation. Combining non-sacrificial subsampling with sacrificial subsampling of in-hive bees may increase the chance of detecting the target matter.

Heavy metals

As for POPs the source of heavy metals coming from industrial stacks, combustion of fossil fuels in road traffic, resuspension of road dust containing heavy metal from brakes and tires, and soil erosion of heavy metal containing soil particles e.g. from streambed and flood plain and from mining dump heaps is irrelevant for bio-indication with honeybee colonies. Unlike POPs, the path is relevant. In the proximity of industrial areas and along roads there is a rather constant exhaust of particles containing heavy metals. Combustion materials from stacks are spread and will deposit over a large area diluting the heavy metal concentration in the air as a function of distance from the source. The chance of detecting heavy metals in the air with honeybees might only be in the vicinity of the industry. Close to roads, resuspended road dust will deposit not far from the source, especially in urban regions due to the microclimate and less wind. In a forest region deposition of airborne particles is accelerated due to the downward wind direction over forests as discussed in Chapter 4. Soil erosion depends on climate conditions, land use and vegetation. Agricultural activities such as ploughing and lowering the groundwater level will increase soil erosion and increase the amount heavy metals in the air. PM will contain no or limited amounts of heavy metals. This path might only be relevant locally. The receptor of the heavy metal containing particles is among others every flower visited by the foraging honeybee. Theoretically the foraging honeybee will collect the most particles, transport them to the hive and accumulate them in the hive and in a non-sacrificial sampler. For bio-indication of heavy metals sacrificial subsampling of in-hive bees has proven to be successful in various studies. Leita et al (1996) demonstrated that heavy metals are present both in and on the bee. In the honeybee the heavy metal concentration depends on the swallowed metals in and on pollen and

nectar, besides the heavy metals which are part of the biological system. Based on the reference / control bees in studies there is a range for heavy metals considered to be normal except for heavy metals not being biologically present in pollen and nectar. Significant exceeded concentrations of completely analysed honeybees are considered to be the result of environmental pollution by heavy metals. Beside the Leita study to my knowledge there is no other studies focussing on the exterior by rinsing the honeybee for the detection of heavy metals. Applying non-sacrificial subsampling will give another dimension to this kind of study as the factor normal concentration range of metals inside the bees is ruled out; non-sacrificial subsampling focuses on particles on the exterior of the bee. The result of non-sacrificial subsampling will be a mixture of particles and pollen. Still there will be an additional pollen factor containing both naturally and possible additional heavy metals. Applying a pollen trap will remove at least part of the pollen from the corbicula and partly rules out the pollen artefact. In study set-ups this must be taken into account. On the other hand, the pollen in the non-sacrificial sample provides important information where the bees foraged. The presence and concentration of heavy metals in nectar and pollen does not by definition represent the presence and concentration of heavy metals in the soil. The uptake of heavy metals is plant species specific and various plants store the heavy metals in vacuoles, roots or other plant parts (Raskin et al., 1994; Clemens et al., 2002). Jones (1987) showed there was no correlation of Cu and Pb in the soil and in nectar.

Airborne plant pathogens

The source of airborne plant pathogens e.g. strands of *Erwinia amylovora* and spores of *Phytophthora spp.* is the infected plant. The path is dissemination via air and the receptor is a plant, receptive for the pathogen. Considerations as for atmospheric depositions of POPs and heavy metals including subsampling methods count as well for the airborne plant pathogens.

Endo- and epiphytic pathogens

Honeybees collect micro-organisms from the petals, along with nectar and pollen. In pollen the pathogen can be both on the surface of the anther or inside the pollen grains. Plant pathogens on leaves and stems are not collected (Kastelein et al 2014). Generally, a plant infection starts with some plants and the infection rate might increase due to different circumstances. Therefore, an unknown fraction of the hive entering foragers will carry the plant pathogen. There is a similarity between honeybee colonies in the field placed in the proximity of a crop possibly infected with the target plant pathogen and those placed in greenhouses.

In the field some of the foragers will forage on the crop and some of the flowers will be infected with the target plant pathogens. In the greenhouse all foragers are restricted to the greenhouse crop. Although greenhouses might cover a large area of several thousand square meters this is still a relatively small site for a honeybee colony and therefore not all potential foragers will collect food. In both cases the fraction of foragers possibly contaminated with the target plant pathogen is unknown. This makes sacrificial sampling of hive-entering bees unreliable. Sacrificial subsampling of in-hive bees is a better alternative. Collected in sufficient numbers, the plant pathogen will be present on many in-hive bees. Also as a consequence the hive-entering bees will have a plant pathogen load at least similar to the in-hive bees. Here the sample size is relevant. The safe sample sizes of in-hive bees exceed the safe sample size for hive-entering bees with a factor 2 (par. 8.2). To avoid the potential restriction of the sample size, non-sacrificial sampling of hive-entering bees can be used. The chance to detect a target plant pathogen depends on the number of bees that passed the non-sacrificial sampler. In greenhouses especially this can be low as mentioned above. In this case a combination sampling methods is recommended.

8.2 Sacrificial subsampling of the honeybee colony

Taking bees and bee's products from a colony affects the colony development and performance anyhow. However due to the buffer capacity of a honeybee colony (par. 1.3.2) a number of bees can be taken for sacrificial subsampling without harming the colony: safe sacrificial subsampling. To my knowledge, there are no studies about threshold numbers of bees that can be taken without affecting the colony significantly. These numbers depend on what age / task cohort is taken and on the status of the colony as for size and time of year. From many years' experience, study practice and collegial discussions, pragmatically I consider 3% of the forager cohort and 1.5% of the total number of bees of a colony (in-hive bees) as number of bees that can be subsampled without affecting the colony significantly. As stated in paragraph 1.5.1 sacrificial subsampling from the hive-entrance will result in a number of bees are likely to carry target matter (both nectar, pollen foragers and bee having collected water on plants) and a number will not (orientating bees). The mean amount target matter depends on an inconsistent subsample composition. By subsampling in-hive bees, this disadvantage is less relevant as due to trophallaxis and in-hive physical exchange, most bees will, because of, carry target matter to a certain extent. Taking bees from the top of the colony results in a sample in which forager bees are over-

represented. This can be done with little disturbance of the colony. Table 1 present an indication of the number of forager bees and in-hive bees that can be sampled safely based on colony size. In the calculation it is assumed that all hive-entering bees are foragers and proportion of foragers in the colony is 25%. The recommended "safe" subsample sizes also imply a maximum frequency of 3-weeks period, the duration of a brood cycle of the honeybee colony.

Table 1. Estimated safe maximal sample size of hive-entering bees and in-hive bees

Colony size	Estimated number of foragers (25%)	Max sample size hive-entering bees	Max sample size in-hive bees
10 000 bees	2500	75	150
15 000 bees	3750	113	225
20 000 bees	5000	150	300
25 000 bees	6250	188	375

Processing bees taken for sacrificial subsampling can be done with the entire bee of by rinsing the bee. Processing entire bees will result in detecting metals or plant pathogens in and on the bee. Rinsing the bee will result in detecting target matter on the bee. In a study by Leita et al. (1996), mentioned in paragraph 8.1 the amount Cd and Zn on the bee's body was significant, showing the accumulation of PM on the bee's hairs. On the other hand, Pb was not detectable in the wash water but was detected in the bee. Pb might have entered the bee via another way. Both ways of processing have pros and cons and the study objective must decide what processing is the best suitable.

Sacrificial sampling of pollen for bio-indication was not part of the studies presented. Fresh pollen can be collected with a pollen trap. The pollen trap is a grid, placed in front of the hive entrance. The bees are forced to pass the narrow holes and lose part of the pollen collected in the corbicula. The efficacy of the pollen trap is variable: 10% (Free, 1967) to 54% (Vaissiere et al., 1996). It depends on pollen pellet size and how tight the pellets are stuck in the corbicula and can therefore result in an over- or underestimation of specific pollen pellets and attribution of specific crops based on pollen harvest in the pollen trap. The disadvantage of collecting pollen is that part of the essential feed of the honeybee colony is taken away and that it will be a mixture of only recently collected materials. Therefore, the collection period of pollen is limited to one or two days per week.

In Table 2, data on sacrificial subsampling, target matter and sample size are presented.

Table 2. Sacrificial subsampling of the honeybee colony

Sacrificial subsampling of bees from flight board of colonies in the field	
Subsample	***Composition of the subsample***
Honeybees - pollen carrying foragers - no pollen-carrying foragers	Hive-leaving and hive-entering foragers + orientating bees foragers having pollen in corbicula nectar foragers + orientating young bees (ratio differs per subsampling)
Subsample size	
maximum sample size*	Depends on the colony size: ≤ 3 % of the forager cohort (estimated on 25% of the colony population). A moderate summer colony is 15000 – 20000 bees Colony 5000 bees: max sample 38 bees Colony 10000 bees: max sample 75 bees Colony 15000 bees: max sample 113 bees Colony 20000 bees: max sample 150 bees Colony 25000 bees: max sample 188 bees
Target matter	
PM particles	Assuming the atmospheric deposition of PM is all over the foraging range, all pollen foragers and part of the no-pollen foragers (100% nectar foragers minus unknown percentage of orientating bees) will have PM adhered to the body.
(plant) pathogens	Assuming the atmospheric deposition of ((plant)) pathogens is all over the foraging range, all pollen foragers and part of the no-pollen foragers (100% nectar foragers minus unknown percentage of orientating bees) will have PM adhered to the body.

Continuing Table 2	
(plant) pathogens	For specific plant pathogens from specific (plant)s: 1. selecting pollen foragers carrying pollen from the (plant) the (plant) pathogen is expected on, will optimise the chance of detecting the pathogen in the subsample. 2. taking no-pollen foragers, the ratio pollen carrying bees from the plant the pathogen is expected on versus all pollen carrying bees will give an indication of the bee than foraged on the specific plant. It is assumed that the ratio pollen collecting and nectar collecting bees on the same source is more or less the same. 3. In case a pollen trap is applied the ratio specific pollen versus all pollen will also give an indication of the number of bees possibly carrying the target micro-organism.

Sacrificial subsampling of in-hive bees in the field	
Subsample	***Composition of the subsample***
Honeybees	In-hive bees: a mixture of age cohorts with age related tasks
Subsample size	
maximum sample size*	Depends on the colony size ≤ 1.5 % of the bees. A moderate summer colony is 15000 – 20000 bees
Target matter	
PM	Due to in-have exchange of matter presumably the majority of the bees will carry target PM. Because of the dilution (par. 1.4) the subsample size may be increased.
Airborne (plant) pathogens	Due to in-have exchange of matter presumably the majority of the bees will carry target airborne (plant) pathogen.

Continuing table 2	
Sacrificial subsampling hive-entering bees in the greenhouse / gauze tents	
Subsample	Hive-leaving and hive-entering bees
Subsample size	Idem categories listed above
Target matter	
Endophytic and epiphytic flower infection	Due to the relatively small foraging area, most bees will forage over the glass house. Depending on the ration diseased / not-diseased flowers more or less bees will carry the pathogen.
Sacrificial subsampling in-hive bees in the greenhouse / gauze tents	
Subsample	In-hive bees
Subsample size	Idem categories listed above
Target matter	
Endophytic and epiphytic flower infection	Due to in-have exchange of matter presumably the majority of the bees will carry target (plant) pathogen. Because of the "dilution" (1.5.3) the subsample size may be increased.

There is no general ruling when to apply sacrificial- and non-sacrificial subsampling and colony samples or apiary samples. This depends on the study objective, site and the target matter. In flow chart I, subsample size for hive-entering bees for a 10,000 bee colony is presented. In case the required sample size < the maximal safe subsample size, both subsampling methods can be applied. In the flow chart (Figure IV) this counts for percentages bees carrying target matter is 50% and 10%. In case the required sample size for sacrificial subsampling > the maximal safe sample size, non-sacrificial subsampling is recommended. Unlike sacrificial subsampling, non-sacrificial subsampling has no limitation concerning number of bees. Non-sacrificial subsampling can be extended in time till at least the required sample size is reached.

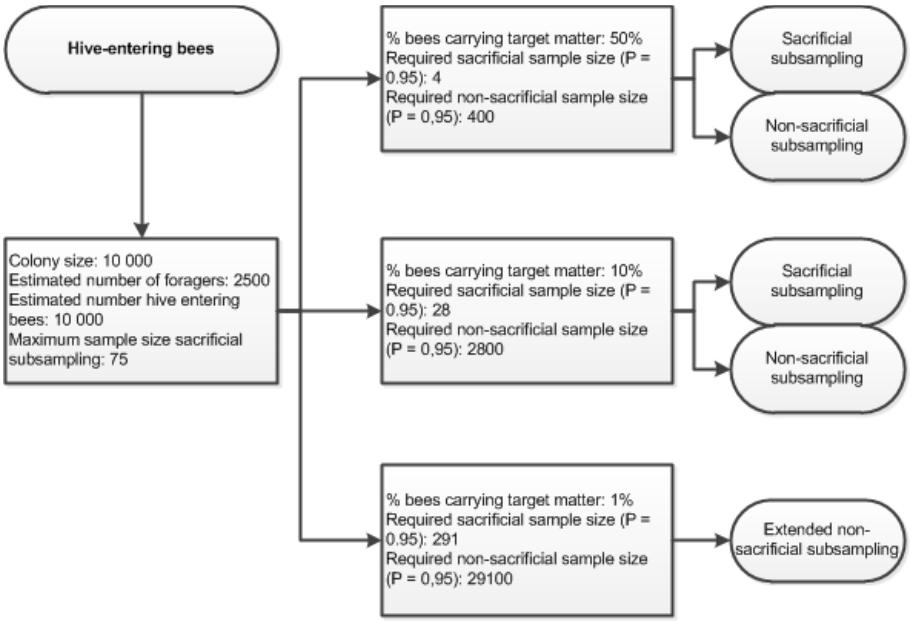


Figure IV. Example of deduction of sampling methods based on percentage bees contaminated

8.3 Non-sacrificial subsampling of the honeybee colony with the Beehold tube

A distinct practical pro of non-sacrificial subsampling is that it can be conducted by non-professional beekeepers and therefore can be applied everywhere apiculture is practiced. Additionally, the Beehold tube can be easily used and the processed material can be used for e.g. in situ lateral flow devices or LAMP techniques for detection of plant pathogens by the grower themselves. There are no ethical cons for non-sacrificial subsampling. Based on the adhered pollen in the Beehold tube, the flowers and possibly the site bees have foraged on can be determined. As about 1% of the target matter is adhered to the PEG, a factor of 100 must be taken into account: 100 bees passing the Beehold tube versus one bee from the flight board. The distinct con of the Beehold tube is that only matter on the bee can be collected and next accumulated and that only part of the materials on the bee's exterior will be accumulated in the Beehold tube. In the studies conducted with the Beehold device so far, the chemical neutral PEG is applied as adherent material. For new applications other specific adsorbent materials can be used even in the same study set-up by using more than one Beehold tube. For example, specific culture

media or general adherence material for bee pathogens like *Paenibacillus larvae*, *Micrococcus plutonius* and adherence material for Nosema spp. as these pathogens can be detected on the honeybee. Also detection of parasites on the bees might be an application of the Beehold tube. The PEG used has proven to work for *Erwinia* spp. (Chapter 5) and pollen (Chapter 5, 6 and 7). As for particles on the bees, non-sacrificial sampling of pollen can be done with the Beehold device. Although compared to the pollen trap the amounts are limited and it might be sufficient for analysis. Contrary to sacrificial subsampling, applying non-sacrificial subsampling an unlimited number of all hive-entering bees during the study can be sampled (Table 3).

Table 3. Non-sacrificial subsample with the Beehold device in the field and in the greenhouse

Subsample	Composition of subsample
honeybees	Hive entering foragers + orientating bees
maximum sample size	unlimited
Target matter	Endophytic and epiphytic flower infection Airborne (plant) pathogens Atmospheric deposition of pollutants e.g. heavy metal containing PM Pollen Pesticides

8.3.1 Estimation of hive-entering bees via the Beehold tube

As a rule of the thumb, the number of hive-entering bees per day is about the same as the estimated number of bees in the colony. The forager cohort is 25% to 40% of the colony, only part of the foragers is actively foraging and a forger makes about 10 trips a day. On average per day, 25% to 50% of the foragers are actively foraging. A strong colony can make up to 35,000 flights per day. It is obvious that the data presented are estimates and depend on food availability, weather conditions and structure of the landscape. This rule of the thumb is confirmed by data of the bee counter in the Beehold device.

8.4.7-steps frame work

Practical aspects of the critical steps in the frame work are based on bio-indication of atmospheric deposition of target matter and on bio-indication of epi- endophytic phyto-pathogens in the field and in greenhouses. In the discussion the order of the critical steps is followed.

8.4.1 Practical aspects of bio-indication of airborne particles to be analysed for heavy metals, POPs and phyto-pathogens

Target matter
Atmospheric deposition of soil particles, combustion particles, resuspension of road particles, PM and airborne phyto-pathogens;

Target matter location
Deposition of target matter occurs over large areas including foraging areas, all flowers can be contaminated;

Location of honeybee colonies
The location of the honeybee colonies is not limited to a defined area. In the field, free flying colonies of an apiary divide themselves over the landscape foraging over more than one crop (paragraph 1.4). Two practical aspects increase the change of successful bio-indication or the target matter:

1. location of the colonies nearby a flowering nectar and pollen yielding fields to enable foraging and the stimulate as many foragers as possible.
2. location of the colonies nearby woods. As demonstrated in the surveillance study 2008 in the Netherlands (Chapter 4) in the proximity of woods deposition increases due to decreasing wind and downward movement of the air because of the colder microclimate conditions and possible by sticky leaves with honeydew.

Sampling colony / apiary
There is no general ruling for individual colony sampling or using pooled samples of an apiary. As a result of large scaled deposition, probably all foragers of all colonies of one apiary are exposed to this atmospheric deposition to more or less of a degree. Analysis of subsamples of individual colonies provides data of the variance between colonies in an apiary. It has to be taken into account that each colony will forages on partly different sites within the foraging area. Analysis of apiary samples (bees of multiple colonies of an apiary in one pooled sample) provides a single outcome of an apiary and the coverage of the foraging area of an apiary is larger that of each individual colony (paragraph 1.4). For the detection of airborne pollutants and plant pathogens, multiple colonies in one apiary should be sampled and possibly be pooled. Multiple colony sampling is preferred to increase the areas bees have foraged on. Sampling of individual bees is relevant where the study objective is the in-hive exchange of plant pathogens or particles.
The number of hives, to be samples in order to obtain a reliable result of pooled apiary samples depends on the variance of the results between

colonies. Based on the Cu, Pb and Cd concentrations and variance per location and sampling period in the study presented in Chapter 2 , the median = 3 (lower quartile = 2; upper quartile = 4; mean = 5; minimum = 2; maximum = 23; n = 49). In practice, three to five colonies is a practical number.

Hive subsample location

In-hive sampling

All in-hive bees will carry to a certain extent target matter where this is brought in by the foragers. The maximum safe sacrificial subsample size depends on the colony strength (paragraph 8.2). Sampling bees from the top of the colony will result in an overrepresentation of older bees and taking bees from the bee-lane will result in a representative sample of the age cohort composition of the colony. Sampling bees from the top causes little colony disturbance.

Hive-entering bees

If target matter is present on the flowers, all foragers will carry it to some extent. The pollen foragers and nectar foragers can be split up by separating bees with pollen in the corbicula and the ones that have no pollen load. The non-pollen carrying bees (nectar foragers + orientating bees) can be split up by weight. The nectar forager cohort bees are heavier than those of the cohort of orientating bees (Gary & Lorenzen, 1976). To discriminate nectar foragers and orientating bees by weight, the subsample must be frozen immediately after collection. Applying sacrificial subsampling, the sample size for safe sampling depends on the colony strength (paragraph 8.2) and on the expected percentage target matter carrying bees (paragraph 1.6). For example, for a 20,000 bees' colony, the max safe sample size = 75, assuming 25 % of the hive-entering bees carry target matter, a minimum of 16 bees must be subsampled. Safe subsampling bees up to 75 increases the change of detecting target matter. Sampling > 75 bees in 3-week periods might affect the colony (par 8.2). To maximize the chance to detect target matter, the nectar foragers and pollen foragers, including the pollen in the corbicula can be pooled.

Sacrificial and non-sacrificial subsampling

For detection of the target matter both sacrificial and non-sacrificial subsampling can be applied. For sacrificial- and for non-sacrificial subsampling the average expected load of target matter per bee determines the subsample size. Non-sacrificial subsampling has no sample size limit. Based on the capture of target matter in the non-sacrificial sampler (e.g. Beehold tube) the number of bees that pass the non-sacrificial sampler should be 100 times more than the required number of

bees taken from the hive-entrance to collect the same amount target matter (Chapter 5).

Subsample analysis
Subsample analysis depends on the target matter and analysis techniques available. As no bees have to be sampled, non-sacrificial subsampling provides the possibility of in-situ analysis.

8.4.2 Practical aspects of bio-indication of endo- and epiphytic phyto-pathogens in the field and greenhouse

Target matter
Endo- and epiphytic phyto-pathogens in the field and greenhouse.

Target matter location
In the field, the sites with potential diseased flowers are a fraction of the potential foraging area. Additionally, only in part of the potential diseased flowers the target phyto-pathogen will be present. Honeybees of one colony forage on multiple food sources (paragraph 1.4) diminishing the probability of bees foraging on flowers with the target plant pathogen. In the greenhouse the target plant pathogen might be present in potentially all flowers visited by the bees. In practice this will be in part of the flowers, especially when the plant disease emerges.

Location honeybee colony
The probability of detecting endo- and epiphytic plant pathogens in the field and in a greenhouse differs significantly. In the field, the probability will increase by locating the honeybee colonies at sites with the possible target plant pathogen. In a greenhouse all foragers visit the flowers available in the greenhouse. To my knowledge there is no information available about the spread of the bees from multiple colonies over the greenhouse. It is assumed that bees from the colonies in the greenhouse visit flowers all over the greenhouse and will be equally exposed to the plant pathogens. The number of foraging bees depends on the potential food availability and influx (paragraph 1.4). The foraging area in a greenhouse is relatively small and therefore the number of foragers will be less compared to in-field colonies. In the field, free flying colonies of an apiary divide themselves over the landscape foraging over more than one crop (paragraph 1.4). A practical aspect increases the change of successful bio-indication of the target matter is to locate the colonies nearby a flowering nectar and pollen yielding fields to enable foraging and to stimulate as many foragers as possible.
In a greenhouse honeybee colonies are placed for pollination, subsampling for phyto-pathogens could be an additional function of the honeybee (Chapter 5).

8.5 Proposed practice

Proposed practices are based on the current state of knowledge and can be improved based on result of new studies as proposed in paragraph 8.6. The proposed practices are discussed for the airborne target matters: airborne PM, airborne particles containing heavy metals, airborne soil particles containing POP's and airborne plant pathogens and for endo- and epiphytic plant pathogens present in flowers. For bio-indication both sacrificial and non-sacrificial subsampling can be applied. As discussed in paragraphs 8.2 and 8.3 the considerations concerning the number of bees that can be taken for sacrificial subsampling is relevant for the decision to subsample sacrificially, non-sacrificially or combined sampling.

8.5.1 PM, airborne particles containing heavy metals or POP's and airborne plant pathogens

Atmospheric deposition of particles (particulate matter, combustion particles, soil particles) and airborne plant pathogens goes over large areas. Matter will be deposited on all flowers and it is likely that all foragers of all colonies in an apiary have on average the same exposure during foraging. Both sacrificial– and non-sacrificial subsampling can be applied. Both sacrificial and non-sacrificial subsampling has proven to work for plant pathogens in a greenhouse. There are no studies to demonstrate effective non-sacrificial subsampling of PM, airborne particles containing POP's and heavy metals.

8.5.2 Endophytic and epiphytic plant pathogen in the flower

In the field, free flying colonies of an apiary divide themselves over the landscape foraging over more than one crop. Therefore, sacrificial subsampling from the flight board significantly reduces the chance of detecting a plant pathogen on a specific crop as only part of the hive-entering bees will carry the target plant pathogen. Due to the in-hive exchange, in-hive sampling increases the chance of finding the pathogen is higher as all bees will, because of in-hive exchange, carry the target (plant)-pathogen. Sacrificial subsampling from the entrance or in-hive is limited for the number of bees. Non-sacrificial subsampling does not have this restriction. Based on the rule of thumb that the number of hive-entering bees per day is about the same as number of bees in the colony, the number of bees passing the non-sacrificial subsampler can be estimated. If this number is not met, extended non-sacrificial subsampling or sacrificial subsampling exceeding the safe sacrificial subsampling size is an alternative.

In a glasshouse where honeybee colonies are placed for pollination, subsampling for plant pathogens is an additional function of the honeybee colony. In a greenhouse the foraging area is restricted to the greenhouse

area. It is assumed that bees from the colonies in the greenhouse visit flowers all over the greenhouse. As in the field an alternative of the sacrificial flight board and in-hive subsampling is the Beehold device.

8.5.3 Individual colonies versus pooled apiary sample

There is no general ruling when to sample individual colonies or make pooled apiary samples. For bio-indication of atmospheric deposition of particles containing POP's, heavy metals or other contaminants and airborne plant pathogens, pooling the samples of individual colonies may be a good practice. All colonies have been exposed to the same deposition on the flowers. Taking the maximum safe sacrificial subsamples per colony and pooling them, increases the change to detect the target matter. The same goes for non-sacrificial subsamples. Bio-indication in the field for epi- and endophytic-pathogens might result in colonies having foraged on flowers with the target pathogen and colonies that haven't. In this case individual colony sampling and analysis might be the best practice. Based on the pollen loads it is possible to pool colonies having foraged on the same target flowers which increase the change for detecting. Bio-indication in the greenhouse implies that all colonies were restricted to one site as is foraging on one crop. Pooling the maximum safe subsample sizes will increase the change of a positive bio-indication of plant-pathogen.

8.5.4 Core numbers of the passive sampling method (PSM) honey-bee colony

The target matter, study objective and study location determines the study set-up. There are best practice conditions, regardless the target matter for a correct use of PSM honeybee colony. These best practice conditions all described and discussed in the previous paragraphs are listed in Table 4: core numbers and conditions of the PSM honeybee colony.

Table 4: Core numbers of the bio-sampling honeybee colony

Honeybee colony	Worker bees	7000 - 35000
	Foragers 25 – 40 % colony population	1750 - 14000
	Resting bees / buffer cohort (10 – 30 % population)	700 - 10500
	Scout bees (10 – 23% foragers)	70 - 2300
	Number of foraging flights / day	7000 - 35000
	Max forage distance nectar	12 km
	Preferred forage distance nectar	≤ 1 km
	Max forage distance pollen	6 km
	Preferred forage distance pollen	≤ 1 km
	Max forage distance water	2 km
	Preferred forage distance water	≤ 1 km
	Forage area nectar in theory	450 km^2.
	Forage area nectar in preferred	≤ 3 km
	Forage area pollen in theory	113 km2.
	Preferred forage area nectar	≤ 3 km
	Nectar load per trip	25 – 40 mg (21 – 33)
	Pollen load per trip	10 – 30 mg
	Estimated annual number of nectar forage flight	4E+6
	Estimated annual number of pollen forage flight	1.25E+6
	Dispersal over landscape	Not homogeneously around hive but directional to most profitable forage sites.
	Estimated percentage of remaining particles after auto-grooming	2 – 4%

Continuing Table 4

Subsampling		
Sacrificial subsampling	Maximal sample size foragers (≤ 3%)	38 - 188
	Maximal sample size in-hive bees (≤ 1.5%)	75 - 375
	Required minimal sample size in case target load is 25% of entire load on bee (D = 0.95 – 0.99)	10 - 16
	Required minimal sample size in case target load is 10% of entire load on bee (D = 0.95 – 0.99)	28 - 44
	Required minimal sample size in case target load is 5% of entire load on bee (D = 0.95 – 0.99)	58 - 90
	Required minima; sample size in case target load is 1% of entire load on bee (D = 0.95 – 0.99)	298-458
Non sacrificial subsampling	Ration n bees sacrificial sampling : n bees passing Beehold tube	1 : 100
Estimate minimal load target matter per bee for detection	Number of particles	4.5
	Weight	0.4 µg

8.6 Further Research

The studies presented are examples of the applications of the passive sampling method honeybee colony conducted with the current state of the knowledge. Working on these studies and this thesis, many questions and ideas came up, both on methodology and applications. Many aspects of the potentially possibilities of bio-sampling by the honeybee colony are still underexposed and underexploited. Improvement of non-sacrificial subsampling and upcoming new analytical techniques with a lower limit of detection (LOD) and limit of quantification (LOQ) can boost the application of the passive sampling method honeybee colony. I mention hereby, without the pretention of being complete:

Improvement non-sacrificial subsampling
The application of non-sacrificial sampling just started and can be improved / customized by

- maximizing the Beehold tube for depth and width;
- applying alternatives for the general PEG coating;
- applying selective coating in the Beehold tube.

Combined sacrificial and non-sacrificial subsampling

The sample size applied at sacrificial sampling are based on the expertise of sampling of the honeybee colony with no significant negative effect on the colony's performance and development. Scientific studies about sacrificial sample sizes of in-hive and forager bees adapted to time of year and status of the colony will add fine-tuning to this aspect of bio-indication.

Combination of sacrificial and non-sacrificial subsampling can provide additional information. For example, in case the number of bees that passed the Beehold tube might be insufficient to detect target matter it will provide information about the food sources by the pollen pallet in the tube. The in-hive bees might provide target matter, collected by and accumulated in the honeybee colony. Additional sacrificial in-hive sampling with the required sample size provides data on the target matter. Design of a smartphone app to decide, based on 1) target matter, 2) atmospheric deposition / in situ contamination or expected presence of endo or epi plant pathogens, 3) colony size, 4) colony status, 5) period of year, where to locate the honeybee colony, the number of colonies at the sampling site, subsampling: sacrificial hive-entering bees, in-hive bees, pollen or non-sacrificial subsampling.

Maximum sample size in-hive and hive entering bees/foragers

For sacrificial sampling of hive entering bees, a practical tool or protocol to separate foragers and non-foragers at the hive entrance, maximizes the sample size of foragers bringing in nectar, pollen foragers, possibly containing target matter and orientating bees. On the practice of dividing the hive entering bees in nectar foragers (water foragers are included), pollen foragers and orientating bees, currently tests are running conducted by the colleagues of the Alterra in collaboration with the PRI bee group.

Possible application of sacrificial-and non-sacrificial subsampling of the honeybee colony

- bio-indication of POP's at storage/dump sites;
- heavy metal deposition of traffic in urban regions;
- airborne dissemination of causes of the zoonosis Q-fever (*Coxiella burnett*);
- atmospheric deposition of fungicidal resistant *Aspergillus* spp causing pneumonia;
- residues of over-year persistent soil- or seed applied systemic pesticides and sprayed pesticides. Depending on the scale and diversity of agricultural areas, pesticides are applied on relatively

small or bigger areas within the foraging areas of a colony. Based on an annual colony need of about 125 kg of nectar, 25 kg of pollen and 25 kg water which have all about the same weight per trip, overall the ratio of foragers for nectar, pollen and water is 5: 1: 1. This indicates that exposure to pesticides is more likely for nectar collecting bees than the ones that forage on pollen and water. Nevertheless, it must be mentioned that pollen has proven to be a good matrix for bio-indication of pesticides.

Applying a non-sacrificial sampling device as a standard equipment of home-apiary honeybee colonies for

- detection of food sources via pollen;
- possible airborne environmental pollution by e.g. POP's heavy metals, radio-active fall out, plant pathogens, zoonosis micro-organisms.
- honeybee diseases e.g. Nosema spp, European Foulbrood (EFB), American Foulbrood (AFB), Deformed Wing Virus and other bee-viruses. Nosema disease is in-hive and between colonies disseminated by spores in the faeces. *Melissococcus plutonius*, the bacterium causing EFB is disseminated in-hive and between colonies as the bacterium contaminated bees cleaning cells with diseased or dead larvae. *Paenibacillus larvae*, the bacterium causing AFB forms persistent spores. Bees cleaning cells containing dead larvae get, as by EFB, contaminated and the bacterium is by in-hive exchange disseminated over the bees.

Applying a non-sacrificial sampling device as a standard equipment of honeybee colonies in greenhouses and at open field sites where honeybee colonies are placed for pollination

The combination of the functions pollination and bio-indication is obvious but nevertheless little or not applied. Further study of the combination Beehold tube / in situ detection of plant pathogens by e.g. LFD or other in situ detection devices may provide the grower with the current health status of the crop. Changing Beehold tubes can be done without knowledge of or expertise in handling honeybee colonies; one of the advantages of passive sampling. Detection of *Erwinia pyrifoliae* in the greenhouse has proven to work. For other plant pathogens the application of non-sacrificial sampling should be studied.

In the open-field situation, as for example in fruit, this set-up has proven to work for the detection of *Erwinia amylovora* in apple orchards. Additionally, based on the pollen pallet of the Beehold tube, the ratio of bees foraging on the crop, the bees are supposed to forage for pollination, can be checked. The latter demands special skills of the fruit grower. By

designing a simple test / reference / smart phone-app images for pollen of the crop to be pollinated. An alternative is sending the Beehold tubes regularly to specialised labs for quick detection of plant pathogens and pollen determination.

Processing pollen pellets
The majority of particles, collected by the forager during food collection, is accumulated in the pollen pellets. Developing techniques e.g. filtering techniques to separate pollen and non-pollen particles might be an alternative for collecting target matter from the hive-entering bee. Trapping pollen for 24 hours in 3-week intervals is considered to be a safe non-sacrificial subsampling method.

Application of non-sacrificial subsampling at sites all over the world where no environmental monitoring infrastructure is available e.g. rural sites
Managed honeybee colonies are present all over the world except at the polar areas. The honeybee colony can be an alternative passive sampling method, especially when non-sacrificial subsampling with e.g. the Beehold tube or other devices is applied. Non-sacrificial subsamples can be sent to specialised laboratories and don't have the restrictions of sending live or dead animals to other countries. Precaution measures at the receiving labs should be taken to prevent unintentional dissemination of pathogens.
Application of the non-sacrificial subsampling of *Apis mellifera scutellata* in Africa and the Africanised honeybee in South America has not yet been studied. Based on the results with the European honeybee *Apis mellifera mellifera*, non-sacrificial subsampling looks promising.

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Appendices

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Summary

Bio-sampling is a function of bio-indication. Bio-indication with honeybee colonies (*Apis mellifera* L) is where the research fields of environmental technology and apiculture overlap. The honeybees are samplers of the environment by collecting unintentionally and simultaneously, along with nectar, pollen, water and honeydew from the flowers or on the leaves, other matter (in bio-indication terms: target matter) and accumulating this in the colony. Collected target matter, in this thesis heavy metals, the plant pathogens *Erwinia pyrifoliae* and *Erwinia amylovora* and the soil pollutant γ-HCH, is collected from the colony by subsampling. Subsampling the honeybee colony is done by taking and killing bees from the hive (sacrificial) or by collecting target matter from the bee's exterior without killing the bee (non-sacrificial). In environmental technology terms the application of the honeybee colony is a Passive Sampling Method (PSM). In this thesis the possibilities and restrictions of the PSM honeybee colony are explored.

Bio-indication is a broad research field with one common factor: a living organism (bio) is applied to record an alteration of the environment (indication). The environment may be small such as a laboratory or big such as an ecosystem. Alterations in the organism may vary from detecting substances foreign to the body to mortality of the organism. In environmental technology the concept Source-Path-Receptor (SPR) is applied to map the route of a pollutant. It describes where in the environment the pollution is, how it moves through the environment and where it ends. This environment is the same environment of all living organisms, ergo also honeybees. Honeybees depend on flowers for their food. In the SPR concept, a flower can be a source, path or receptor. Along with collecting pollen, nectar, water and honeydew, target matter is collected by honeybees. Each honeybee functions as a micro-sampler of target matter in the environment, in this case the flower. Each honeybee is part of a honeybee colony and in fact the honeybee colony is the bio-sampler. The honeybee colony is a superorganism. The well-being of the colony prevails over the individual honeybee. Food collection is directed by the colony's need. Foragers are directed to the most profitable food sources by the bee dance and food exchange (trophallaxis). The result of this feature is that mainly profitable sources are exploited and poor food sources less or not at all. During the active foraging period hundreds to thousands of flowers are visited daily. The nectar, pollen, water and honeydew plus the unintentionally collected target matter is accumulated in the honeybee colony. In order to obtain target matter the colony must be subsampled. This is done by picking bees from the hive-entrance (hive-entering bees) or inside the hive (in-hive bees) and processing them for

analysis (sacrificial). This is the most commonly applied method. However, it is possible to subsample the colony without picking and processing the bees by collecting target matter from the hive-entering bee's exterior (non-sacrificial). For non-sacrificial subsampling of the honeybee colony the Beehold device with the sampling part Beehold tube has been developed. The results of bio-indication with honeybee colonies are qualitative and indicative for follow up study (Chapter 1).
Six bio-indication studies with honeybee colonies for bio-indication of heavy metals, the plant pathogens *Erwinia pyrifoliae* and *Erwinia amylovora* and the soil pollutant γ-HCH are presented. Chapter 2 describes how the concentration of eighteen heavy metals in honeybees fluctuate throughout the period of July, August and September (temporal) at the study sites: the city of Maastricht, the urban location with an electricity power plant in Buggenum and along the Nieuwe Waterweg at Hoek van Holland (spatial). A number of the metals have not been previously analysed in honeybees. To study whether honeybees can be used for bio-indication of air pollution, the concentrations of cadmium, vanadium and lead were compared to concentrations found in honeybees. The honeybee colonies were placed next to the air samplers. Only significant differences of metal concentrations in the ambient air also show in honeybees. This was the case with vanadium in ambient air and honeybees. The spatial and temporal differences of cadmium and lead were too futile to demonstrate a correspondence (Chapter 3). In a national surveillance study in 2008 the concentration of eighteen metals in honeybees has been analysed. The results showed a distinct regional pattern. Honeybees in the East of the Netherlands have higher concentrations of heavy metals compared to the bees in the West. Besides regional differences local differences were also recorded. An approximate description of the land use around 148 apiaries (> 50% agriculture, > 50% wooded area, > 50% urban area and mixed use) indicated the impact of land use on metal concentrations in honeybees. In areas with > 50% wood significantly higher concentrations of heavy metals were detected (Chapter 4). Subsampling of the honeybee colonies in Chapter 2, 3 and 4 was done sacrificially. In the studies presented in Chapter 5, 6, and 7 the honeybee colonies were subsampled non-sacrificially or simultaneously non-sacrificially and sacrificially. The plant pathogen *E. pyrifoliae* causes a flower infection in the strawberry cultivation in greenhouses. In greenhouse strawberry cultivation honeybees are applied for pollination. In Chapter 5 the combination pollination / bio-indication by honeybee colonies is studied. This proved to be a match. *E. pyrifoliae* could be detected on in-hive bees prior to any symptom of the infection in the flowers. In the Beehold tube, the bacterium was detected at the same

time as the first tiny symptoms of the infection. In Chapter 5 the principles on which the Beehold tube is based are presented and discussed. The plant pathogen *E. amylovora* causes fireblight in orchards. The combination pollination / bio-indication has also been applied in this study performed in Austria in 2013. It is known that *E. amylovora* can be detected on honeybees prior to any symptom in the flower or on the fruit tree. A fireblight outbreak depends on flowering period, humidity and temperature. In 2013 no fireblight infection emerged in the orchards where the study was performed. Therefore, the bacterium could not be detected on the honeybees. γ-HCH (Lindane) is one of the soil pollutants in the Bitterfeld region in Saxony-Anhalt in Germany. It is the result of dumping industrial waste around the production locations. Although γ-HCH is bound to soil particles there is a flux to groundwater and surface water. Consequently, the pollution may end up in the sediments of the streambed and flood plains. The study objective was to investigate the hypothetic route of γ-HCH from polluted soil (source), via soil erosion and atmospheric deposition (route) to the receptor (flowering flowers) by detecting γ-HCH in the Beehold tube. Although on average over 17000 honeybees passed through the Beehold tube daily for a maximal period of 28 days, no γ-HCH has been detected. The pollen pattern in the Beehold tube revealed where the bees collected the food (Chapter 7).

The application of the honeybee colony has pros and cons. Distinctive pros are many micro samplers, the extensive collection of matter (both food and target matter) and the accumulation in the colony. For successful bio-indication with honeybee colonies, determining factors are: the target matter, location of the target matter, distance between target matter and the honeybee colony, individual or pooled subsampling, the minimal sampling frequency and sample size, and sacrificial or non-sacrificial subsampling applied solely or in combination. Taking bees from a colony impacts upon the colony's performance and consequently the passive sampling method. Based on a long-years' experience and inter-collegial discussion it is stated that 3% of the forager bees (hive-entering) and 1.5% of the in-hive bees can be sampled safely without impacting upon the colony. This restriction does not apply when carrying out non-sacrificial subsampling of the honeybee colony (Chapter 8).

Performing bio-indication with honeybee colonies has more applications than have been exploited so far. Further research can make a change. In particular I mention here the combination of pollination and bio-indication and the application of non-sacrificial subsampling solely or in combination with sacrificial subsampling.

Everywhere Apiculture is practiced (all over the world except the polar areas) bio-indication with honeybee colonies can be applied in a simple, practical and low cost way.

Nederlandse samenvatting

Deze thesis gaat over het honingbijenvolk (*Apis mellifera* L) als bio-indicator. Bio-indicatie met bijenvolken is het onderzoeksgebied waar milieutechnologie en apicultuur elkaar overlappen. De bijen zijn monsternemers van het milieu door in het veld passief, simultaan met het verzamelen van nectar, stuifmeel, honingdauw en water, ander materiaal (in bio-indicatie-termen: doelmateriaal) in de bloemen of op de bladeren te verzamelen en vervolgens samen te brengen in het bijenvolk. In deze thesis worden de mogelijkheden en beperkingen van het honingbijvolk als bio-monsternemer in kaart gebracht.

Bio-indicatie omvat een breed onderzoeksveld met één gemeenschappelijke factor; een levend organisme (bio) wordt gebruikt om een verandering in het milieu aan te tonen (indicatie). Dit milieu kan klein zijn zoals in het laboratorium of groot wanneer het gaat om veranderingen van ecosystemen. De verandering in het organisme kan variëren van het aantonen van lichaamsvreemde stoffen in of op het organisme tot mortaliteit van het organisme. Voor het in kaart brengen van vervuilingsstromen wordt in de milieutechnologie het concept bron-pad-ontvanger (source-path-receptor) gebruikt. Het beschrijft waar in de leefomgeving zich vervuiling bevindt, welk pad door de leefomgeving wordt gevolgd en waar de vervuiling terecht komt. Deze leefomgeving is de leefomgeving van alle organismen en dus ook honingbijen. Honingbijen zijn voor hun voedsel volledig aangewezen op bloemen. Bloemen kunnen in milieutechnologietermen zowel de bron als het pad als de receptor zijn. Bij het verzamelen van stuifmeel, nectar, water en honingdauw wordt onbedoeld ook doelmateriaal meegenomen. Elke honingbij functioneert als een micro-monsternemer van doelmateriaal dat in het milieu, in dit geval in bloemen, terecht komt. Elke honingbij is onderdeel van een bijenvolk en feitelijk is het bijenvolk de monsternemer. Het bijenvolk is een superorganisme waarbij het belang van het volk prevaleert boven het belang van het individu. Het verzamelen van voedsel wordt gestuurd door de behoefte van het volk. Met de bijendans en voedseluitwisseling (trophallaxis) in het volk, worden haalbijen naar de beste voedselbronnen (dracht in bijenteelt termen) gedirigeerd. Dit heeft tot gevolg dat vooral rijke drachten benut worden en arme drachten niet of minder bezocht worden. In de actieve periode van het bijenvolk worden dagelijks honderden tot (tien)duizenden bloemen bezocht. Nectar, stuifmeel, water en honingdauw plus wat er eventueel aan doelmateriaal onbedoeld meegenomen is, komt samen in het bijenvolk. Het bijenvolk wordt daarom in milieutechnologie termen beschouwd als een passieve monstername methode (Passive Sampling Method). Om doelmateriaal uit het bijenvolk te verkrijgen wordt het volk bemonsterd. Dit gebeurt door bijen van de vliegplank of uit de

bijenkast te nemen en deze te doden voor het onderzoek (sacrificial). Dit is de meest gebruikelijke methode. Het is echter ook mogelijk bijen te bemonsteren zonder ze te doden door materiaal van het exterieur af te halen bij het binnengaan van de bijenkast (non-sacrificial). Hiervoor is de Beehold tube ontwikkeld. De resultaten van bio-indicatie met het bijenvolk zijn kwalitatief en indicatief voor vervolgonderzoek (Chapter 1).
In deze dissertatie worden zes onderzoeken gepresenteerd waarbij het bijenvolk gebruikt wordt voor bio-indicatie van zware metalen, de plantpathogenen *Erwinia amylovora en Erwinia pyrifoliae* en de bodem-verontreiniging γ-HCH (Lindaan). In Chapter 2 wordt beschreven hoe de concentraties van achttien metalen in de honingbij in de periode juli, augustus, september op drie verschillende locaties (stad Maastricht, landelijke omgeving in Buggenum met een elektriciteitscentrale in de buurt en Hoek van Holland aan de Nieuwe Waterweg) kunnen fluctueren. Een aantal van deze metalen is nog niet eerder in bijen geanalyseerd. Om te bepalen of honingbijen ook gebruikt zouden kunnen worden voor bio-indicatie van luchtvervuiling met cadmium, lood en vanadium zijn de concentraties van genoemde metalen in honingbijen vergeleken met de gegevens van deze metalen in de lucht. De luchtmetingen werden uitgevoerd naast de bijenstanden. Uitsluitend voor vanadium werd een positief verband vastgesteld. Alleen bij grote verschillen en hoge metaal concentraties in de lucht is een positief verband aan te tonen. Voor lood en cadmium waren de verschillen in tijd en ruimte in de bijen en in de lucht te klein om een verband aan te tonen (Chapter 3). Bij een landelijk surveillance-onderzoek in 2008 werden achttien metalen in honingbijen onderzocht. De resultaten laten een duidelijk regionaal patroon zien waarbij de metaalconcentraties in bijen in Oost Nederland hoger zijn dan in West Nederland. Naast regionale verschillen werden ook lokaal verschillen vastgesteld. Een globale beschrijving van het landgebruik (> 50% agrarisch, > 50% bos, > 50% bebouwing en gemengd gebruik) rond 148 bijenstanden gaf duidelijke aanwijzingen dat landgebruik invloed heeft op de concentratie metalen in de honingbij; deze is in gebieden met veel bos hoger dan in agrarische of bebouwde gebieden (Chapter 4). De bemonstering van de bijenvolken in de onderzoeken in Chapter 2, 3 en 4 zijn uitgevoerd met "sacrificial sampling". In Chapter 5, 6 en 7 wordt onderzoek gepresenteerd waarbij de volken geheel of gedeeltelijk bemonsterd zijn met "non-sacrificial sampling". De plantpathogene bacterie *Erwinia pyrifoliae* veroorzaakt een bloeminfectie en richt schade aan in de aardbeienteelt onder glas. In deze teelt worden honingbijen gebruikt voor de bestuiving. In Chapter 5 wordt de combinatie van bestuiving en bio-indicatie onderzocht. Dit blijkt een werkzame combinatie te zijn. *Erwinia pyrifoliae* kon op de bijen in het volk aangetoond worden

voordat er symptomen van de ziekte in de bloemen te zien waren. Met de Beehold tube werd de bacterie aangetoond bij het begin van de eerste symptomen van de ziekte. In genoemd Chapter 5 wordt het principe van de non-sacrificial sampler Beehold tube uitgelegd en bediscussieerd. De plantpathogene bacterie *Erwinia amylovora* veroorzaakt bacterievuur in fruit. De eerdergenoemde combinatie van bestuiving en bio-indicatie met non-sacrificial sampling is ook toegepast bij dit in 2013 in Oostenrijk uitgevoerde onderzoek. Het is bekend is dat *Erwinia amylovora* al op bijen te detecteren is voordat er symptomen in de fruitbloei en fruitbomen te zien zijn. De uitbraak van bacterievuur is afhankelijk van bloeiperiode, vocht en temperatuur. In 2013 trad geen bacterievuur op in het gebied waar het onderzoek uitgevoerd is en kon de bacterie dan ook niet aangetoond worden. In het gebied rond Bitterfeld in Saksen Anhalt Duitsland, is γ-HCH (Lindaan) een van de bodemverontreinigingen. Het is het resultaat van het dumpen van industrieel afval rond de productieplaatsen aldaar. Hoewel γ-HCH aan bodemdeeltjes gebonden is, is er ook een stroom van deze verontreiniging via het grondwater naar het oppervlaktewater. Deze verontreiniging kan daardoor ook in het sediment van het oppervlaktewater en in de uiterwaarden terecht komen. Het onderzoek was erop gericht om na te gaan of deze verontreiniging via de bron (verontreinigde bodem), pad (bodemerosie en atmosferische depositie) en receptor (bloeiende bloemen) met de non-sacrificial sampler Beehold tube aangetoond kon worden. Hoewel gemiddeld ruim 17000 bijen per dag op deze manier bemonsterd werden met een maximale duur van 28 dagen, werd geen γ-HCH gevonden in het materiaal dat achterbleef in de Beehold tube. Wel kon aan de hand van het stuifmeel in de Beehold tube een beeld gevormd worden waar de bijen het voedsel verzamelden (Chapter 7).

Het bijenvolk, gebruikt als bio-indicatie methode, heeft naast voordelen, zoals het grote aantal micromonsternemers, het intensieve verzamelen van materiaal in de bloemen en het accumuleren in het volk, ook beperkingen. Voor bio-indicatie met bijenvolken zijn de bepalende factoren bij de opzet en uitvoering van een studie: 1) wat is het doelmateriaal?; 2) waar bevindt zich het doelmateriaal?; 3) waar is de locatie van het bijenvolk ten opzichte van het doelmateriaal?; 4) moeten individuele volken of bijenstanden bemonsterd worden?; 5) hoeveel en hoe frequent worden bijen uit het volk genomen (sacrificial) en 6) kan sacrificial– of non-sacrificial monstername of een combinatie van beide bemonsteringen uitgevoerd worden? Het wegnemen van bijen uit een volk beïnvloedt het gedrag en de taakverdeling binnen het volk en daarmee ook de passieve monstername door het bijenvolk. Gebaseerd op jarenlange ervaring wordt gesteld dat maximaal 1,5% van het volk en 3%

van de haalbijen per drie weken bemonsterd kan worden zonder het volk te schaden. Deze beperking geldt uiteraard niet voor het non-sacrificial bemonsteren.
Bio-indicatie met bijenvolken biedt meer mogelijkheden dan tot nu toe benut worden. Vervolgonderzoek kan hier verandering in brengen. Met name noem ik hier de combinatie bestuiving / bio-indicatie voor het aantonen van plantpathogenen en het toepassen van de non-sacrificial monstername alleen of gecombineerd met sacrificial monstername. Aan de hand van de verwachte mate van verontreiniging of een verwacht optreden van een bepaalde microbiële plantenziekte, de mate waarin materiaal van de bijen op de Beehold tube overgedragen wordt, de nauwkeurigheid van de analysemethode (Limit of Detection) en de drachtomgeving kan een inschatting gemaakt worden van het aantal bijen dat nodig is om een bepaalde stof of micro-organisme op de bijen te detecteren en of bio-indicatie met sacrificial of non-sacrificial sampling of een combinatie van beide monstermethoden succesvol kan zijn. Deze aspecten worden bediscussieerd in Chapter 8.
In gebieden waar geen milieutechnische infrastructuur is en wel een bijenhouderij, en dit is over heel de wereld het geval behalve in de poolgebieden, kan bio-indicatie met bijenvolken toegepast worden. Dit biedt mogelijkheden voor eenvoudige praktische bio-indicatie in ontwikkelingslanden.

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About the Author

Jozef J.M. van der Steen was born on 23 January 1952 in Chaam. His life-long learning route started at the secondary school MULO-A at the St Marie boarding school in Huybergen from 1964 to 1968. After that he started the professional educations: secondary professional education clinical chemistry followed by higher professional education medical microbiology completed in 1973. Subsequently in part time education the following studies were completed: applied bachelor medical biology (1980), teaching credential (1980), teacher of apiculture (1980) and at the Open University quality management and change management (2000). The study Environmental Sciences at the Open University was completed with the MSc degree in 2009. The PhD project that led to this thesis started in 2013.

The professional career started in April 1975 with a laboratory technician position at the apicultural research station Ambrosiuhoeve in Hilvarenbeek. From 1980 till 1981 he was also a part time biology teacher at the Dr Struycken Institute. In time his position developed into doing apicultural research. His special interest goes out to research topics linking the environment to honeybees and bumblebees. This implies studies ranging from protocol development for first tier tests for pesticides to field monitoring studies and the impact on the honeybee colony's vitality.

Since 2013 he is member of the EC of Coloss, the European network of apicultural researchers, since 2013 co-chair of the Coloss CSI pollen working group and chair of the non-apis working group of the International Commission of Pollinator - Plant Relationships.

Further to his professional positions he values social involvement. He was member of the board of Sparrenhof, a Catholic vacation location of underprivileged children from 1997 – 2001. Besides playing as an amateur violoncellist in the Oosterhouts Sympfonie Orkest, he also chairs this orchestra.

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