Toxic potency and effects of diffuse air pollution

Timo Hamers

Promotor:

Prof Dr JH Koeman
Hoogleraar in de Toxicologie
Wageningen Universiteit

Co-promotor:
Dr AJ Murk
Universitair Hoofddocent, Leerstoelgroep Toxicologie
Wageningen Universtiteit

Promotiecommissie:
Dr TCM Brock
Alterra Green World Research, Wageningen

Prof Dr NM van Straalen
Vrije Universiteit Amsterdam

Prof Dr HA Udo de Haes
Rijksuniversiteit Leiden

Prof Dr M Scheffer
Wageningen Universiteit

Toxic potency and effects of diffuse air pollution

Timotheus HM Hamers

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The air is precious to the red man,
for all things share the same breath
– the beast, the tree, the man –
they all share the same breath.

Chief Seattle, 1854

Voor mijn vader
die zo trots was op zijn kinderen

Contents

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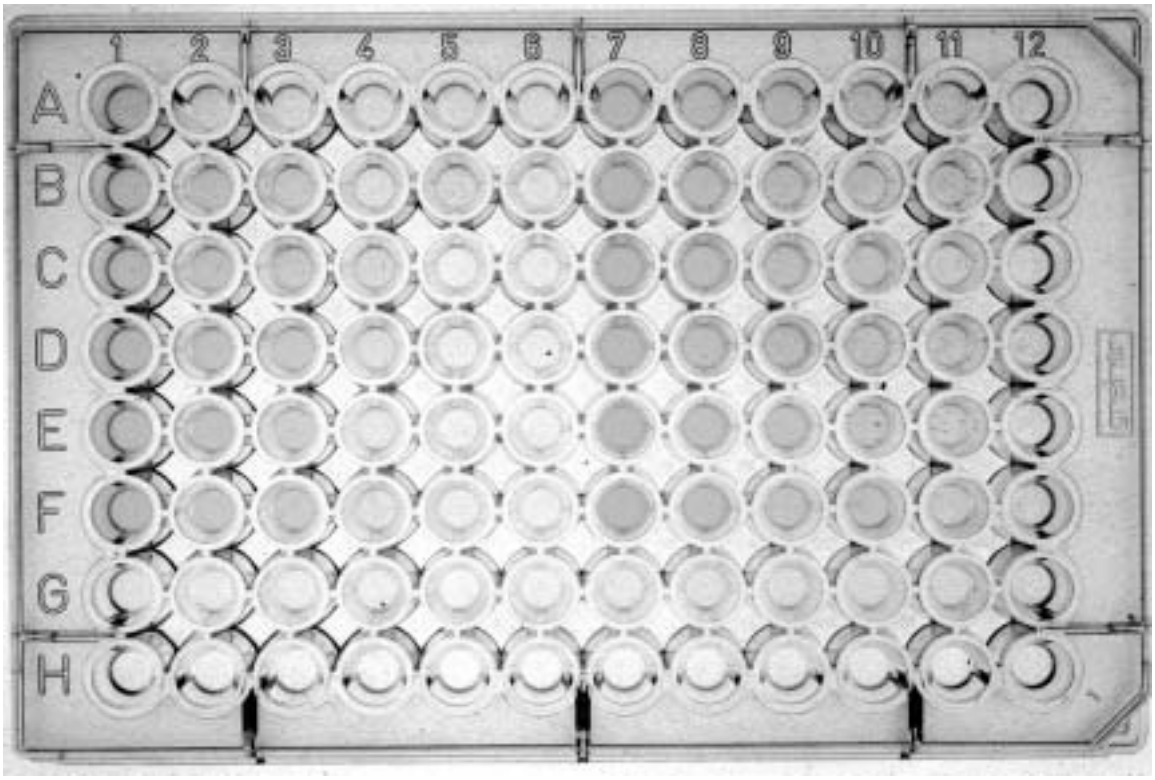
General introduction



The *umu*-assay is a bio-assay in which genetically modified bacteria specifically to respond DNA damaging compounds (genotoxicants). It is performed in small volumes (270 µl) in a so-called microtiter 96-wells plate. In the example plate shown above, the genotoxicity of two model compounds is demonstrated. The color intensity is a measure for the genotoxicity added to each well. For each column 1 to 6, rows A to F are filled with replicate concentrations of the first genotoxicant. Declining concentrations in columns 1 to 6 are reflected by a decreased color intensity from left to right. Similarly, columns 7 to 12 contain declining concentrations of a second genotoxicant. Rows G and H contain control (no genotoxicants added) and background (no bacteria added) wells, respectively.

Diffuse air pollution – the problem

In terms of tons per year, most wastes from our industrialized society are emitted to the air compartment. For instance, the Dutch emission registration estimated that 285 kton of organic compounds (excluding CH_4) were emitted to the air compartment in The Netherlands in 1999 compared to 35 and <1 kton to the water and soil compartment, respectively (CCDM, 2000). Most of the organics emitted to the air originate from dispersed sources including traffic (42%), industries (22%), households (11%), and the energy sector (7%). Another important source of diffuse air pollution is agriculture, which contributes significantly to emission of pesticides, NH_3, CH_4, and N_2O. Generally, only emissions of known compounds are registered or analyzed in monitoring programs. For instance within the yearly emission registration, assessments are made of the emissions of the acidifying, eutrophicating, and greenhouse gasses NH_3, NO_x, SO_x, CH_4, N_2O, and CO_2. Additionally, emissions are assessed for toxic compounds including heavy metals, pesticides, and halogenated and non-halogenated aliphatic and aromatic hydrocarbons (*e.g.* CCDM, 2000). However, the mixture of air pollutants consists of many unknown compounds, such as degradation products and reaction products resulting from combustion or atmospheric photomodification processes. Knowledge on the chemical identity and toxicity of the constituents of the mixtures is still rather limited.

Due to atmospheric transport, air pollution can reach relatively remote areas, which are not in the close vicinity of a notorious site of emission and which are therefore supposed to be clean. For instance, studies from our own laboratory demonstrated mutagenic activity and tumor promoting effects of airborne particulate matter collected in rural and background locations in The Netherlands (Alink *et al.*, 1983; Van Houdt *et al.*, 1987; Heussen, 1991). Applying trajectory analyses, Van Houdt *et al.* (1987) further demonstrated that relatively undiluted pockets of air with high mutagenic activity originated from the industrial Ruhr area in Germany and the Antwerp harbor area in Belgium. De Raat and De Meijere (1988) confirmed that a substantial part of the mutagenic airborne activity in an urban area in The Netherlands probably originated from long-range transport. Similarly, atmospheric presence and deposition of pesticides has been demonstrated in remote areas far away from their site of application. In samples of air and wet deposition collected in the Sierra Nevada mountains, organophosphate insecticides were found that probably had been applied ±200 km further in the fruit orchards of Central Valley, CA (Zabik and Seiber, 1993). Persistent organic chlorine

pesticides appear in arctic air, probably after long-range atmospheric transport by polluted aerosols and by global distillation (Biddleman *et al.*, 1993; Allsopp *et al.* 1999).

In summary, diffuse air pollution can be characterized as a very complex mixture that is omnipresent as a "gray veil". It is emitted by numerous national and international sources, which can impossibly be traced individually. It has a heterogeneous composition in time and space and contains a substantial fraction with an unknown chemical identity and toxicity.

Toxicity of air pollution

Episodes of increased air pollution that coincided with increased mortality have focused attention on the potency of air pollution to affect human health (see overviews by Goldsmith and Friberg, 1977 and ACE, 2000). Such disastrous incidents often occurred in river valleys under extraordinary atmospheric conditions of temperature inversion in combination with foggy weather. Emissions to the air compartment were trapped in the fog, and could not be diluted into a larger volume of air due to the stagnant atmospheric conditions preventing air circulation to higher atmospheric layers. The first smog (smoky fog) episodes were reported in London at the end of the 19^{th} century. As a result of increased coal burning in winter, citizens were exposed to yellow/black clouds of smoke particles and SO_2 that covered the city for days. In December 1952, the most severe London smog period resulted in 3500 to 4000 excess death numbers ascribed to bronchitis, broncho-pneumonia and heart diseases (Goldsmith and Friberg, 1977). Especially the Los Angeles basin is known for a different type of smog consisting of ozone and other reactive oxidants including peroxyacyl nitrates (PAN) that cause respiratory and eye irritation. This so-called photochemical smog arises when hydrocarbons and NO_X emitted by industry and traffic emissions are trapped in fog and react together under the influence of sunlight.

In The Netherlands, Biersteker (1966) performed the first studies on the effects of air pollution on human health. The author demonstrated that urban air pollution was a very minor cause of mortality and morbidity in Rotterdam, even during the smog episode of 1962. Much focus has further been given to traffic emissions. For instance, Brunekreef *et al.* (1983) demonstrated that blood levels of lead in inner city children were higher than in suburban children, probably as a consequence of elevated exposure to lead emitted by traffic. Leaded gasoline was banned in The Netherlands in 1996, but traffic emissions still contribute significantly to air pollution. In a later paper, Brunekreef *et al.* (1997) demonstrated that

especially diesel exhaust particles might cause reduced lung functions in children living near motorways.

With respect to ecological effects of airborne pollution, most studies have focused on lichens and plants. Already in 1859, declining lichen flora in England was attributed to air pollution. Based on the presence and absence of lichens on bark trees, a scale was developed in the 1970s by which sites could be allocated to one of 11 zones, indicating mean winter airborne concentrations of SO_2 (Hawksworth and Rose, 1970; 1976). Plant exposure to ethylene has been known to cause growth abnormalities, senescence, and reduced growth since the beginning of the 19^{th} century (reviewed by Abeles *et al.*, 1992). Later studies on air pollution have focused on the phytotoxicity of acidifying gasses as SO_2, HF and HCl (reviewed by Guderian, 1977) and photochemical oxidants as ozone and PAN (reviewed by Guderian, 1985) causing chlorosis, necrosis and reduced growth. Airborne herbicides such as 2,4-D can also negatively affect plants, especially after long-term exposure (Tonneijck and Van Dijk; 1993).

Risk characterization of complex mixtures

Traditionally, a toxicological risk characterization of mixtures is based on an evaluation of the individual constituents of the mixture. For air pollution, assessing the risk of the individual constituents is only feasible in situations where one or a few pollutants occur in overwhelming concentrations, as was the case with SO_2 during smog periods and with Pb along highways. For the complex mixture of diffuse air pollution, however, such an approach is virtually impossible, given the unknown chemical identity and toxicity of a substantial fraction mixture. Moreover, it neglects the fact that many compounds still contribute to the overall toxic potency of the mixture of air pollutants, although their individual levels are too low to be detected or to cause any effects. For the purpose of an adequate risk assessment of diffuse air pollution, a better approach would be to assess the integrated toxic potency and the overall effect of the air polluting mixture by measuring biomarkers that specifically respond to major categories of constituents of airborne pollution.

Biomarkers are xenobiotically induced alterations in cellular or biochemical components or processes, structures, functions or behavior that are measurable in a biological system or sample (Committee on Biological Markers of the National Research Council, 1987). These alterations are considered as early warning signals for irreversible adverse effects at higher exposure levels. Originally, biomarkers were measured *in vivo* in ecologically relevant

organisms living in contaminated areas to demonstrate internal exposure to pollutants and to indicate the magnitude of the organism's response (*e.g.* McCarthy and Shugart, 1990; Fossi and Leonzio, 1994). However, some of these xenobiotically induced alterations can also be measured in *in vitro* bio-assays in which cultured bacteria or cells or isolated enzymes are exposed to relevant environmental samples. In this latter case, biomarkers are used to qualify and to quantify the toxic potency of pollutants in the environment. In the present thesis, biomarkers have been measured both in *in vitro* bio-assays and in *in vivo* exposed organisms. They have been classified according to the objective for which they have been applied, *i.e.* exposure assessment or effect assessment.

Objectives and approach of the present study

Because knowledge on the environmental impact of diffuse air pollution is mainly restricted to individual compounds affecting plants, the present study focuses on a broader hazard characterization of airborne pollution and its possible effects on ecologically important species. Three objectives have been defined for the present study:

1. to determine the overall exposure to diffuse air pollution by measuring biomarker responses to relevant environmental samples;
2. to determine possible (early-warning) effects of diffuse air pollution by analyzing biomarkers in ecologically relevant exposed organisms;
3. to develop a strategic research concept for a toxicological risk characterization of diffuse air pollution based on overall exposure and effect assessment.

To meet the objectives of the study, the following approach has been applied. Within the exposure assessment (**objective 1**), a hazard characterization was made by qualifying (hazard identification) and by quantifying (hazard quantification) the integrated toxic potency of diffuse air pollutants to affect specific and non-specific endpoints in small-scale *in vitro* bio-assays. Environmental samples have been collected of airborne particulate matter and rainwater. Additionally, chemical analyses were performed to validate and to explain the bio-assay results and to identify guiding compounds. Within the effect assessment (**objective 2**), possible changes in biochemical and physiological endpoints have been quantified in organisms exposed to airborne pollution in the field. To distinguish between routes of exposure to deposited pollutants, herbivorous voles and carnivorous shrews were studied. The research described in this thesis does not pretend to present a complete risk characterization of the diffuse air pollution in the Netherlands. It explores the applicability and limitations of a

biomarker approach to characterize the risk of diffuse air pollution in addition to a chemical approach. Based on the experiences of this study, a conceptual model (**objective 3**) has been proposed to study the risk of diffuse air pollution.

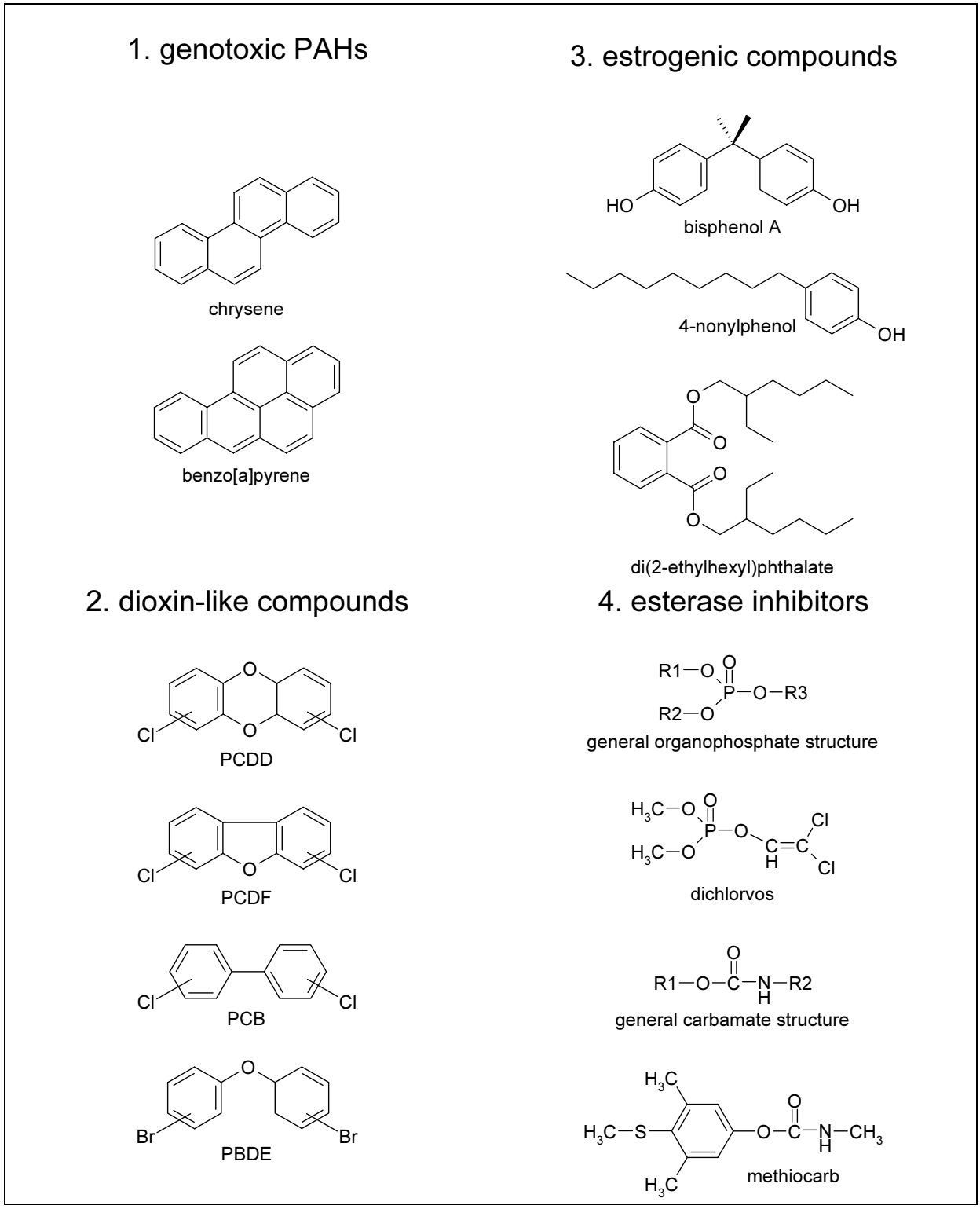


Figure 1: Structures of representative compounds belonging to the groups of air pollutants studied in the present thesis. PAH: polycyclic aromatic hydrocarbon; PCDD: polychlorinated dibenzo-*p*-dioxin; PCDF: polychlorinated dibenzofuran; PCB: polychlorinated biphenyl; PBDE: polybrominated diphenyl ether.

Selection of toxicologically relevant groups of airborne pollutants

Exposure and effect assessments have been made for four groups of airborne toxicants. They were selected because earlier studies pointed out that they are relevant airborne pollutants or because little is known about their atmospheric presence (Figure 1).

1. Genotoxic DNA damaging compounds have been demonstrated in the atmosphere in The Netherlands by our own group in the 1980s and early 1990s. Consistent research has shown that airborne particulate matter with mutagenic activity can be found even in relatively remote areas probably originating from industrial areas abroad (Alink *et al.*, 1983; Van Houdt *et al.*, 1987; Heussen, 1991). Polycyclic aromatic hydrocarbons (PAHs) are a major class of airborne mutagens. Anthropogenic PAH emissions are mainly produced during incomplete combustion of fossil fuels, and are therefore ubiquitous in the air compartment (NRC, 1972; Baum, 1978, IARC, 1983). Atmospheric presence of PAHs in The Netherlands has extensively been demonstrated (De Raat, 1994). Traffic (36%), industry (27%) and households (17%) are the main contributors to the 709 tons of PAH emissions to the air compartment that were estimated for the year 1999 (CCDM, 2000).
2. Persistent organic pollutants with a dioxin-like mode of action are another class with ubiquitous environmental distribution. They all belong to the group of polyhalogenated aromatic hydrocarbons (PHAHs), including major classes as polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). Acute and (sub)chronic effects of PHAH exposure include weight loss, disrupted hormone homeostasis, hepatotoxicity, teratogenicity, neurotoxicity, immunotoxicity and tumor promotion (Kimbrough, 1974; McConnell, 1985; Safe 1990; De Vito and Birnbaum, 1994). PCDDs and PCDFs are undesired side-products of incomplete combustion in the presence of chlorine-containing compounds. Their atmospheric presence, distribution, long-range transport, and deposition have been widely documented (see overview by Zook and Rappe, 1994). PCDD and PCDF emissions have drastically decreased in The Netherlands over the past decade due to the remediation of waste incinerators and to the ban on leaded gasoline to which chlorine containing lead-scavenging were added to prevent coating of the engine and exhaust system with lead. Given their very persistent chemical properties, PCBs and PBDEs have been commercially produced for industrial application on a wide scale. From the early 1930s, PCBs have especially been used as fire-resistant dielectric fluids in capacitors and transformers (Brinkman and De Kok, 1980). Swedish EPA estimated that

by now one third of the produced volume of PCBs has entered the environment (Bernes, 1999). Although the industrial use of PCBs has been banned almost world-wide, they are still entering the environment by leaking and volatilization from old systems or from waste dumps (Allsopp *et al.*, 1999). PBDEs are a relatively new group of PHAHs, which are used as flame-retardants in plastics used for electronic devices, building materials and textiles. Their annual global production was estimated to be 40 ktons in 1992 (IPCS, 1997). Strandberg *et al.* (2001) demonstrated the atmospheric presence of PBDEs even in remote areas and found a significant correlation between airborne concentrations of PCBs and PBDEs.

3. Compounds with estrogenic activity have hardly been studied in the atmosphere so far, although the environmental presence of endocrine disrupting compounds is attracting considerable attention both in The Netherlands and internationally. By mimicking the female hormone estrogen, xeno-estrogens can cause effects on reproductive success. Estrogenic activity has been described for many xenobiotics, including surfactants as alkylphenoles, plasticizers as phthalates, organochlorine insecticides as o,p'-DDT and drins, and bisphenol-A, a compound used in the manufacture of polycarbonates and coatings (*e.g.* Tyler *et al.*, 1998). Estrogenic potency has also been demonstrated for some of the mutagenic and dioxin-like compounds described above, such as benzo[a]pyrene (PAH) and PBDEs (Legler *et al.*, 2002a; Meerts *et al.*, 2001). So far, estrogenic potency in the atmosphere has only been tested in three rainwater samples within the Dutch National Survey on Estrogenic Substances (LOES). These samples exhibited estrogenic activity that was about twice that of surface water from large rivers. The responsible compounds could not be determined yet (Vethaak *et al.*, 2002).
4. Esterase inhibiting organophosphate and carbamate insecticides made up 70% of the total amount of insecticides sold in The Netherlands in 1998 (RIVM/CBS, 1999), whereas Brouwer and Van Berkum (1998) estimated that insecticide use in The Netherlands is one of the highest in Europe in terms of kg per ha of arable land. Organophosphates and carbamates form an important part of the mixture of airborne pesticides, because they are toxic to a broad spectrum of species and are efficiently removed from the atmosphere by wet deposition. The atmospheric presence of organophosphates and carbamates in The Netherlands has been extensively described by Fleverwaard (1993), the Province op South-Holland (1994) and Baas and Duijser (1997).

Exposure assessment

Selection of bio-assays

In correspondence with the four selected toxicologically relevant groups of air pollutants, four bio-assays have been selected that specifically respond to the known mode of action of these groups. Therefore, they allow qualification and quantification of the toxic potency of the airborne pollution. A fifth non-specific bio-assay was added, which responds to a wide range of toxicants. For screening purposes, such a general toxicity assay deserves an important role, because it may respond to compounds that are missed by chemical analyses or more specific bio-assays. All selected bio-assays have been developed or adapted to be performed in small volumes, so that environmental samples could be concentrated to a maximum when tested in the bio-assays.

Specific bio-assays

The presence of genotoxicants, dioxin-like compounds, and estrogenic compounds in atmospheric samples has been demonstrated and quantified using three different reporter gene assays, *i.e.* the *umu*-, the DR-CALUX and the ER-CALUX assay, respectively. The general principle of reporter gene assays (Figure 2) is based on a genetically modified bacteria strain or cell line in which a so-called reporter gene has been implemented (*e.g.* Koeman, 1998). Expression of this gene construct is only triggered when a specific interaction takes place between toxicants and a cellular component present in the cytoplasma or in the nucleus. After transcription of the reporter gene the reporter messenger RNA is subsequently translated into a reporter enzyme of which the activity can easily be quantified.

1. The *umu*-assay was developed by Oda *et al.* (1985) and responds specifically to genotoxic compounds. DNA damage induces expression of the *umu*-gene, which belongs to the bacterial SOS regulatory network consisting of many genes with increased expression in case of DNA-damage (see Walker, 1984). In the *umu*-assay, a strain of *Salmonella typhimurium* bacteria is used that is stably transfected with the plasmid pSK1002 carrying a lacZ gene under control of the *umu* regulatory region. The lacZ reporter gene encodes for proteins with β-galactosidase activity that can metabolize a colorless substrate (ONPG) into a yellow-colored product (o-nitrophenol). Thus, the induced enzyme activity is quantified by an increase in optical density, which is a direct measure for the exposure level of the bacteria to genotoxic compounds in the medium.

Based on an adjusted protocol developed by Reifferscheid *et al.* (1991) who first applied the *umu*-assay in a 96-wells microtiter plate, standardized and validated protocols are currently available for the determination of the genotoxic potency of surface water and wastewater samples (DIN, 1996; ISO, 2000). To distinguish between pollutants that can directly cause DNA damage and pollutants that can only cause DNA damage after metabolization, the assay is performed in the absence and in the presence of a rat liver homogenate (S9-mix) that can metabolize indirect genotoxicants into their DNA damaging metabolites. Responses of the *umu*-assay to direct and indirect genotoxicants in the environmental samples have been expressed in equivalent concentrations of the model genotoxicants 4-nitroquinolin-oxid (4-NQO) and 2-aminoanthracene (2-AA), respectively (Chapter 2). Equivalent concentrations were calculated by interpolating the responses of unknown samples in the calibration curve of the model compound.

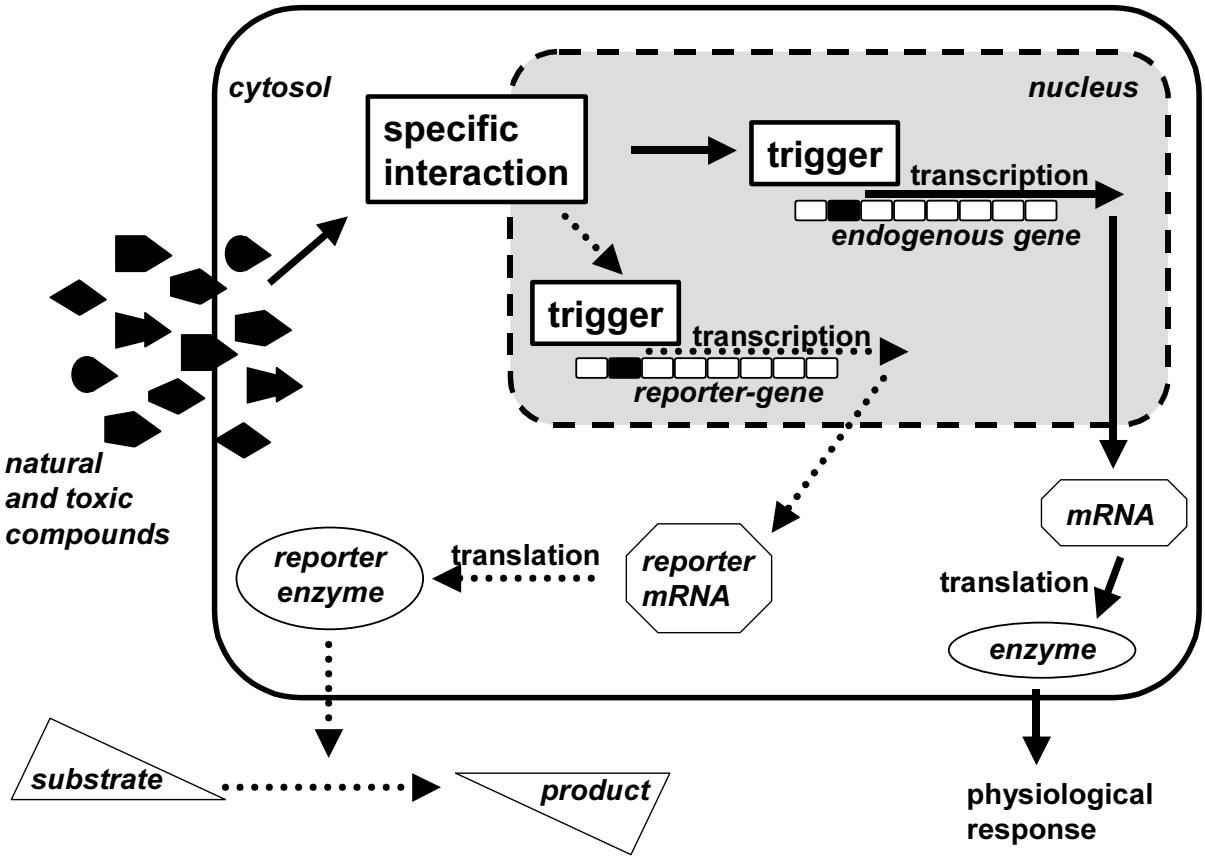


Figure 2: General principle of reporter gene assays: specific interaction between natural or toxic compounds with a cellular component induces expression of the reporter gene. This specific interaction may be activation of a specific receptor as in the CALUX-assays or damage to DNA as in the *umu*-assay. The solid arrow indicates the natural cellular response to this interaction, the dotted arrow the reporter gene response. Expression of the reporter gene leads to the production of reporter enzymes of which the activity can easily be quantified, for instance by a change in color or in light production.

2. The DR-CALUX (Dioxin-Responsive Chemical-Activated LUciferase gene eXpression) assay was developed by Aarts *et al.* (1995). It responds specifically to compounds that bind to the arylhydrocarbon receptor (AhR), such as dioxin-like compounds. Upon binding, the AhR-ligand complex is activated and translocated to the nucleus where it specifically binds to dioxin-responsive elements (DREs) and induces or inhibits the transcription of genes under control of DREs. The most investigated AhR-mediated response is an induced cytochrome P450-1A (CYP1A) synthesis, which is often measured as an increased EROD-activity (see further).

   The DR-CALUX assay makes use of a hepatoma cell line of rat (H4IIE) stably transfected with a vector containing the luciferase gene of firefly (*Photinus pyralis*) under transcriptional control of the DRE. Luciferase activity is quantified by measuring the light production in presence of the substrate luciferin. This light production is a direct measure for the exposure level of the cells to AhR agonists.

   In addition to the use for dioxin-like compounds, the DR-CALUX assay can be used to determine AhR activation by readily biodegradable compounds such as PAHs by varying incubation times. Relatively persistent compounds such as dioxins and PCBs still induce high AhR-agonistic activity after 48 h of exposure, whereas most of the biodegradable AhR-agonists show a peak in activity after 6 h of exposure and then become metabolized. DR-CALUX responses to environmental samples have been expressed as benzo[a]pyrene equivalent (BEQ) concentrations and as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent (TEQ) concentrations for experiments with incubation times of 6 and 48 h, respectively (Chapter 2).

3. The ER-CALUX (Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression) assay was developed by Legler *et al.* (1999). It responds specifically to pseudo-estrogenic compounds that can mimic the activity of the female sex hormone estradiol. Similar to estradiol, pseudo-estrogens bind to the estrogen receptor (ER), inducing an activation of the receptor. After dimerization, activated ER-ligand complexes are translocated in pairs to the nucleus where they specifically bind to estrogen receptor elements (EREs) in the DNA, inducing transcription of genes under control of the ERE.

   The ER-CALUX assay makes use of a human breast adenocarcinoma cell line (T47D), in which a plasmid is stably transfected with the luciferase gene under control of three EREs. Exposure of the cells to estrogenic compounds results in luciferase synthesis that is quantified by measuring the light production in presence of the substrate luciferin,

similar as in the DR-CALUX. Using the major endogenous estrogen 17β-estradiol (E2) as a standard compound, ER-CALUX responses to environmental samples have been expressed as estradiol equivalent (EEQ) concentrations (Chapter 5).

4. Although the esterase inhibition assay is also a specific bio-assay, it is not making use of the reporter gene principle. Instead, the principle of this assay is based on the specific binding of organophosphate and carbamate insecticides to the enzyme acetylcholinesterase (AChE). By blocking AChE these insecticides inhibit the hydrolization of the neurotransmitter acetylcholine (ACh), leading *in vivo* to a longer life-time of ACh in the synaptic gap during neurotransmission and thus to an overstimulation of the post-synaptic membrane. Eventually this may cause paralysis and death of the organism.

   The *in vitro* esterase inhibition assay used in this thesis was especially developed for determination of the toxic potency of environmental (rainwater) samples as described in Chapter 3. It makes use of a homogenate of honeybee heads containing AChE and other esterase-enzymes that can be inhibited by organophosphate and carbamate insecticides. This homogenate is incubated with esterase-inhibitors extracted from rainwater samples. The remaining esterase activity is determined by measuring the hydrolysis rate of the model substrate N-methylindoxyl acetate (MIA) into acetate and the fluorescent hydrolysis product N-methylindoxyl. Thus, the reduction in fluorescence relative to the uninhibited control is a direct measure for the exposure of the enzymes in the homogenate to esterase inhibiting compounds. Dichlorvos was used as a model compound in all experiments and esterase inhibiting potency of the rainwater samples has been expressed as dichlorvos equivalent (DEQ) concentrations (Chapters 3-5).

Non-specific bio-assay

5. The non-specific bioluminescence-assay applied in this study is similar to the Microtox-assay first described by Bulich *et al.* (1979). As described in Chapter 4, the assay protocol was specifically adapted to allow fast and small volume testing. The assay makes use of naturally bioluminescent marine *Vibrio fischeri* bacteria, which emit visible light under normal life conditions as a consequence of a series of metabolic reactions that are intrinsically tied to cell respiration. Bioluminescence intensity may be regarded as a general "health status" of the bacteria, because damage to any part of the cell involved in metabolic processes, results in decreased bioluminescence. As this metabolic damage may

occur at any level of cell organization, such as cell wall, cell membrane, electron transport chains, enzymes, cytoplasm composition, and energy status, the assay can indicate the toxic potency of a broad-spectrum of compounds with different modes of action (see for instance Kaiser and Palabrica, 1991).

Effect assessment

For the effect assessment, *in vivo* biomarkers have been measured in small mammals to indicate possible responses to traffic related airborne pollutants (Chapter 6). Selected biomarkers were DNA damage measured as bulky aromatic DNA adducts in heart, lung and liver, induced hepatic EROD and PROD activity, and disturbed vitamin A homeostasis.

Levels of bulky aromatic DNA adducts have been measured as a biomarker for internal exposure to PAHs: increased levels indicate that the small mammals have been exposed to increased levels of PAHs that have been metabolized into DNA binding metabolites and therefore pose an increased risk for DNA damage. The formation of bulky DNA adducts and their role in carcinogenesis has been reviewed by Godschalk (1999). After uptake, PAHs are metabolized by cytochrome P450 and epoxide hydrolase. During this metabolization, many PAHs are not detoxicated but transformed into highly reactive intermediates with high affinity for covalent binding to DNA. In case these adducts are not removed by DNA repair, they may hinder accurate DNA replication and can lead to basepair substitutions and subsequent mutations (Dennisenko *et al.*, 1996). DNA adducts have been measured using ^{32}P-postlabeling technique (Reddy and Randerath, 1986), which is based on digesting isolated DNA into separate nucleotides and selectively labeling the nucleotide-adduct complexes with [γ-^{32}P] phosphate. Labeled adducts are then separated from each other by chromatographic procedures and quantified by phosphor imaging techniques.

Hepatic EROD and PROD activity have been measured in liver microsomes as a biomarker for effects of PAHs and PHAHs (Burke *et al.*, 1977). Increased EROD (ethoxyresorufine-O-dealkylation) activity was measured as an indicator of CYP1A synthesis, which is an AhR-mediated response to PAHs (Owens, 1977) and planar PHAHs (Safe, 1992). PROD (pentoxyresorufine-O-dealkylation) activity was measured as an indicator of induced CYP2B synthesis as a response to non-planar PHAHs (Safe, 1994).

Vitamin A levels have been measured because PHAHs can induce developmental effects on fetuses and newborns that resemble symptoms of vitamin A deficiency. Numerous animal studies have confirmed that PHAHs indeed alter vitamin A homeostasis (Zile, 1992).

Therefore, hepatic retinol (RE) and retinyl-palmitate (RP) levels were measured as a biomarker for PHAH-induced effects in the present study, with RE and RP being the active form and the storage form of vitamin A, respectively. Especially a decrease in hepatic RP storages is known to be a sensitive indication for exposure to PHAHs. Zile (1992) suggested that a depletion in hepatic vitamin A stores is the result of reduced esterification of RE into RP, and by higher RE levels in blood circulation as a result of an increased demand by peripheral tissues due to an increased metabolism and elimination of circular vitamin A.

Study sites

In a densely populated country as The Netherlands with many industrial activities and a tight network of infrastructure, it is difficult to select appropriate field sites to study the exposure and possible effects of diffuse air pollution. As we were interested in the omnipresent gray veil of pollution we selected background sites for our studies that were not in the immediate vicinity of specific point sources. Given earlier studies on airborne mutagens (Alink *et al.*, 1983; Van Houdt *et al.*, 1987; Heussen, 1991) and pesticides (Fleverwaard, 1993; Province of South-Holland, 1994), it was clear from the beginning that unexposed control sites do not exist in The Netherlands. Therefore, we compared exposure and effects at background sites to exposure and effects at sites that were notoriously exposed to relatively high levels of diffuse air pollution. In this way, pollution at background locations could be compared to a worst-case scenario. Background sites studied in this thesis were located in the nature conservation area De Regulieren near Culemborg and at the shore of Lake Nieuwkoopse Plassen in Noorden. Because traffic is a major contributor to emissions of (genotoxic) airborne particulate matter including aromatic hydrocarbons, highly exposed sites were selected next to the very busy highway A2 near Everdingen. With respect to pesticide emissions, highly exposed sites were selected in two areas that were dominated by different intense agricultural practices, *i.e.* greenhouse horticulture in the Westland area (Naaldwijk) and flower bulb cultivation in the so-called Bulb-area (Hillegom).

Outline of this thesis

To meet the objectives of the project, several studies have been conducted with respect to exposure assessment (Chapters 2-5) and effect assessment (Chapter 6) of diffuse air pollution.

In Chapter 2, the toxic potency of traffic emissions has been determined in airborne particulate matter (APM) collected in springtime during two consecutive years. Responses of the *umu*-assay and DR-CALUX assay to APM extracts were discussed taking account of differences in sampling sites and prevailing wind directions during sampling.

Chapter 3 describes several experiments to determine the most sensitive enzyme-substrate combination that can be used in a small-scale bio-assay to quantify the esterase inhibiting potency of rainwater samples. Experiments have been performed with the model inhibitor dichlorvos, two types of esterase enzymes and two types of substrates. The optimized bio-assay has been applied in a pilot study (Chapter 4) and in a year-round monitoring program (Chapter 5) for esterase inhibiting potency of rainwater. Additionally, the bioluminescence assay (Chapter 4) and ER-CALUX assay (Chapter 5) were performed on the same rainwater samples, and bio-assay results were compared to concentrations of pesticides that were chemically analyzed in simultaneously collected rainwater samples. Differences in bio-assay responses and accompanying pesticide concentrations have been analyzed and discussed in Chapter 5 with respect to sampling location and sampling season.

Chapter 6 describes a field study with carnivorous common shrews (*Sorex araneus*) and predominantly herbivorous bank voles (*Clethrionomys glareolus*) that are exposed *in situ* in an expectedly decreasing gradient of traffic emission with an increasing distance to the highway. After dissection, DNA adduct levels were determined in heart, lung, and liver. In addition, hepatic EROD and PROD activity were measured as well as hepatic RE and RP levels, and internal exposure levels of heavy metals were measured in kidney. Results from this study have been discussed with respect to the differences in air pollution levels at the different sites determined in Chapter 2, and to the different positions that both species occupy in the food chain.

Finally, the results and conclusions from the preceding Chapters 2-6 are summarized in Chapter 7. In addition, a strategic research concept is proposed for risk characterization of diffuse air pollution that is concluded from the experiences with the biomarker methodology applied in the preceding chapters. Finally, a pointed overview is given of the conclusions drawn from the research described in this thesis.

Chapter 2

The application of reporter gene assays for the determination of the toxic potency of airborne particulate matter

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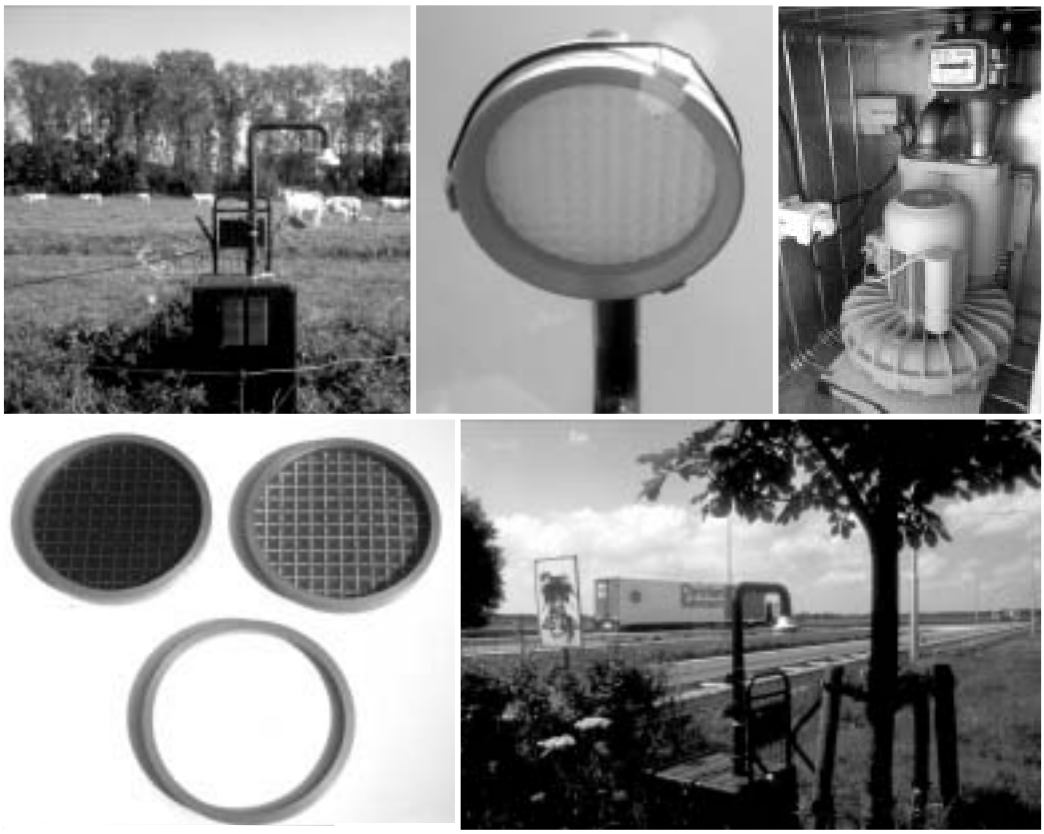
Timo Hamers
M Dennis van Schaardenburg
Emiel C Felzel
Albertinka J Murk
Jan H Koeman



Clockwise explanation of the pictures, starting from the upper left corner: (1) High-volume air sampler (GROMOZ) at site BACKGROUND, *i.e.* nature conservation area De Regulieren near Culemborg, (2) head of the GROMOZ during sampling with quartz fiber filter supported by a metal grating, (3) inner look in the GROMOZ showing the pump (center), the gas flow meter (top) and the automatic time switch (left), (4) GROMOZ at site HIGHWAY, *i.e.* next to highway A2 near Everdingen, (5) quartz fiber filters before sampling (bottom) and after 24 hours of sampling at highway location (left top) and at background location (right top) with grid pattern caused by the supportive grating that prevents the filter from being sucked into the pump.

Abstract

Diffuse air pollution consists of a mixture of numerous compounds. It is emitted by many widely distributed sources and is omnipresent due to atmospheric transport. Risk assessment of the complex mixture of air pollutants on the basis of the toxicity of the individual compounds is not possible yet, because the chemical identity and/or toxicity of the constituencies of a substantial fraction are unknown. In addition, possible combination effects are disregarded because no adequate procedures are available to integrate toxicity data of such complex mixtures. In the present study, an approach has been developed to assess the toxic potency by using *in vitro* bio-assay techniques. Genotoxicity was assessed in the *umu*-assay, a reporter gene assay using a strain of *Salmonella typhimurium* stably transfected with a plasmid (pSK1002) carrying the SOS-gene *umuC* fused to the reporter gene lacZ. Activation of the arylhydrocarbon receptor (AhR) was assessed in the DR-CALUX-assay, using a stably transfected H4IIE hepatoma cell line containing a plasmid for a luciferase gene under transcriptional control of dioxin-responsive elements. In the spring of 1998 and 1999, samples of airborne particulate matter (APM) were collected with a high volume sampler next to a highway and in a natural conservation area. Both assays proved to be applicable to quantify genotoxic pollutants and biodegradable AhR agonists, such as polycyclic aromatic hydrocarbons (PAHs), in APM extracts. Bio-assay results showed that a basic airborne toxic potency is always present in a relatively background area in The Netherlands, even under clean northerly (*i.e.* marine) wind directions. This basic toxic potency was only less than one order of magnitude smaller than under polluted conditions, as regularly found down-wind from a highway and incidentally with easterly winds. APM pollution during easterly wind is attributed to long-range transport of polluted air packages, because it is independent of sampling location and has a different composition than highway-related pollutants, consisting of more S9-persistent compounds. Trajectory analysis proved to be a helpful and transparent tool to explain the origin of APM samples, confirming that at least one of the polluted easterly APM samples descended from the industrial Ruhr area in Germany. Dioxin-like compounds contributed negligibly to the AhR agonistic potency of APM. Airborne pollutants with genotoxic and/or PAH-like characteristics form an undesired mutagenic risk, which will be evaluated in further *in vivo* studies.

Introduction

The long-term aim of the program of which the present study forms part is to assess the possible impact of diffuse air pollution on terrestrial ecosystems. In the present paper emphasis has been put to measurement of exposure levels by using a range of bio-assay techniques.

In terms of tons per year, wastes from our industrialized society currently are mainly emitted to the air. This includes widely studied compounds as NH_3, NO_x, SO_x, CH_4 and CO_2 and thousands of halogenated and non-halogenated hydrocarbons as polycyclic aromatic hydrocarbons (PAHs). For instance, according to the Dutch emission registrations (Berdowski *et al.*, 1997), a total amount of 375 kton of organic compounds (excluding CH_4; including halogenated and non-halogenated and aliphatic and aromatic compounds) was calculated to be emitted into the air in a small country such as the Netherlands in 1995, of which 368 kton were defined as volatile (*i.e.* vapor pressure <10 Pa at 20°C). For comparison, 16 kton of organic compounds were calculated to be emitted to the water and about 1 kton towards the soil compartment. The compounds emitted to the air originate from different sectors, of which traffic (42%), industry (22%), refineries and energy plants (11%) and households (9%) are the most important (Berdowski *et al.*, 1997).

Air pollution is not locally restricted to its site of emission. In gaseous form or bound to particles >10 μm, air pollutants can easily be dispersed over long distances through the atmosphere. For instance, mutagenic activity, as well as the presence of many chemical groups including (chlorinated) aromatic pesticides and persistent organic compounds have been demonstrated in air and deposition samples relatively far away from polluting sources (Van Houdt *et al.*, 1987; Biddleman *et al.*, 1993; Simonich and Hites, 1995; Hüskes and Levsen, 1997).

Therefore, air pollution is a diffuse pollution, which is characterized as being

- omnipresent (*i.e.* covering the globe as a gray veil of polluted air)
- originally emitted from many different national and international sources
- composed of a mixture of many compounds at often low concentrations
- composed of not only parent compounds, but also of transformed products.

The chemical identity and/or the toxicity of the constituents of a substantial fraction of the complex mixture of air pollutants are unknown. Presently, attempts are made in the European Community to make an inventory of produced existing chemicals (EINECS-list: European Inventory of Existing Chemical Substances), many of which are emitted as pollutants to the

environment. Next to that, a list is made of newly produced chemicals (ELNECS-list: European List of New Chemical Substances). So far, approximately 102000 chemicals have been registered. For only 5000 of these substances, data and documentation exist to evaluate the most obvious, potential effects on human health and/or the environment, whereas for only a few hundred sufficient thorough knowledge is available for a proper risk assessment (Bro-Rasmussen *et al.*, 1996). However, air pollution not only consists of registered parent compounds, but also of their metabolites and degradation products, whose chemical identity and toxicity are not known. In addition, no adequate procedures are available to integrate toxicity data of such complex mixtures, so that an individual risk assessment of the constituents of air pollution disregards possible combination effects. Therefore, risk assessment for diffuse air pollution on the basis of the toxicity of the individual components is impossible due to a lack of knowledge on chemical identity and toxicity of the pollutants.

For the purpose of an adequate assessment of diffuse air pollution, a better approach would be to measure its integrated toxic potency by using specific bio-assays that are able to identify and quantify relevant toxic endpoints. Two reporter gene assays were selected which respond specifically to groups of chemicals known or suspected to be predominant constituents of polluted air. The assays were optimized and validated for determination of the toxic potency of samples of airborne particulate matter (APM), which was collected in a natural conservation area and on an obvious polluted site along the highway. Road traffic was selected as a main source of air pollution, containing many different (polycyclic) aromatic hydrocarbons, which are possible genotoxic and/or Arylhydrocarbon receptor (AhR) mediated toxicants.

Genotoxicity of APM was tested in the *umu*-assay (Oda *et al.*, 1985; Chapter 1), a reporter gene assay using a *Salmonella typhimurium*-strain, which is stably transfected with the plasmid pSK1002. This plasmid carries a lacZ gene under control of the *umu* regulatory region. The *umu*-gene is part of the SOS regulatory network, which is induced by DNA-damage and whose expression affects the mutagenic consequences of a variety of DNA-damaging treatments. In the transfected *S. typhimurium*-strain, DNA damage caused by mutagens generates an inducing signal (SOS-response), which activates the *umu*-operon and leads to the production of a protein with β-galactosidase activity by the lacZ gene. After incubation of the produced enzymes with o-nitrophenyl-β-D-galactopyranoside (ONPG), the induced β-galactosidase activity is quantified by measuring the optical density (λ=420 nm) of the yellow-colored product o-nitrophenol.

So far, application of the *umu*-assay for screening the genotoxic potency of environmental samples has mainly been restricted to aquatic samples. Preliminary results with air samples have only been described by Lu *et al.* (1991) and Whong *et al.* (1986). As far as we know, this is the first publication applying the *umu*-assay for environmental monitoring of air quality.

The potency of APM to activate the Arylhydrocarbon receptor (AhR) was tested in a reporter gene assay using a hepatoma cell line of rat (H4IIE) stably transfected with a vector containing the luciferase gene of firefly (*Photinus pyralis*) under transcriptional control of the Dioxin Responsive Elements (DRE) (Aarts *et al.*, 1995; Chapter 1). Exposure of the cells to AhR agonists activates the production of luciferase, which is quantified by measuring the light production after addition of the substrate luciferin. The assay is further referred to as the DR-CALUX (Dioxin-Responsive Chemical-Activated Luciferase gene Expression). Recent DR-CALUX experiments in our laboratory indicated that AhR activation by easily biodegradable compounds such as several polycyclic aromatic hydrocarbons (PAHs) could be distinguished from the response to relatively persistent compounds such as dioxins and other polyhalogenated aromatic hydrocarbons (PHAHs). In the test system used, PAH-induced luciferase production can already be measured after 6 h of exposure but is no longer detectable after 24 h of exposure, when most of the PAHs and the produced luciferase have been metabolized. Relevant PHAHs are much more persistent and still induce luciferase production after 24 h and longer.

Finally, in a pilot-experiment the applicability has been tested of the intercellular communication (IC) inhibition assay with a few samples. Inhibited communication between cells across gap-junctions is supposed to be involved in the tumor promotion phase in multistage carcinogenesis (Yamasaki, 1995). This assay may be a valuable extension of the set of bio-assays selected for the assessment of the toxic potency of diffuse air pollution.

Materials and methods

Sampling of Airborne Particulate Matter

Active APM sampling was performed over a quartz-filter with high volume samplers (Gromoz; Vrins *et al.*, 1985). Samplers were especially developed for the purpose of outdoor sampling, containing a gas flow meter for reading the volume of air sampled. The filter holder was situated at 1.60 m above ground level and collected air at a flow rate similar to human

inhalation (1.1 m/s). Filters (Schleicher and Schuell; QF20; ∅ 150 mm) were pre-washed by overnight standing and additional swinging in methanol, dichloromethane and hexane (HPLC-quality), consecutively. Methanol and dichloromethane were obtained from Labscan (Dublin, Ireland) and hexane from Rathburn (Walkerburn, Scotland). Filters were conditioned for at least 24 h in a exsiccator with saturated K_2CO_3 (air humidity of 45%), and weighed just before use. Wind directions and other weather conditions were recorded at the start and at the end of each sampling period, which usually was 24 h (Table 1). Samples were collected during working days at three sites in The Netherlands (Figure 1):

1. Site PILOT was located 10 m south of highway A12 next to traffic point Grijsoord near Wolfheze (November 1997). Traffic intensity during working days in 1998 was estimated to be $63*10^3$ vehicle passages per day (vpd) (Transport Research Center, Heerlen-NL, personal communication).
2. Site HIGHWAY was located 10 m east of highway A2 next to exit Everdingen (1998 and 1999). Traffic intensities were estimated to be $93*10^3$ and $98*10^3$ vpd in 1998 and 1999, respectively (Transport Research Center, Heerlen-NL, personal communication).
3. Site BACKGROUND was located 5 km east of highway A2 in De Regulieren (1997, 1998 and 1999), a natural conservation area (181 ha) near Culemborg, NL. It is characterized by non-manured grasslands with extensive cow grazing alternated with willow bushes and poplar bushes. It is supposed to be exposed to background levels of diffuse air pollution.

Sampling was performed under conditions of different prevalent wind directions, enabling discrimination between local and regional sources of pollution.

Both *umu* and DR-CALUX assays were optimized and validated for the measurement of the genotoxic and AhR activating potency air samples collected at sites PILOT and BACKGROUND in 1997. Next, the assays are applied on a set of 68 APM samples collected at sites HIGHWAY and BACKGROUND from April to July in 1998 and 1999. The characteristics of all samples described are given in Table 1.

Sample preparation

After sampling, filters were conditioned in the exsiccator (45% air humidity), weighed and stored at –20°C. Before extraction, half of the filter was cut into pieces. All pieces were collected in a stainless steel filter and extracted in a Tecator Soxtec-apparatus (Höganäs, Sweden). Samples were boiled and rinsed for 1 h at 160°C with a (1:1 v/v) combination of acetone and dichloromethane (Labscan; Dublin, Ireland; AR and HPLC quality, respectively).

Extracts were filtered and dried over 1g Na_2SO_4-columns for removal of solid parts from filter extracts. Solvents were evaporated at 37°C under a gentle flow of N_2-gass. Just before dryness, 50 µl of dimethylsulfoxide (DMSO; Acros 99.9% pure, Geel-Belgium) was added.

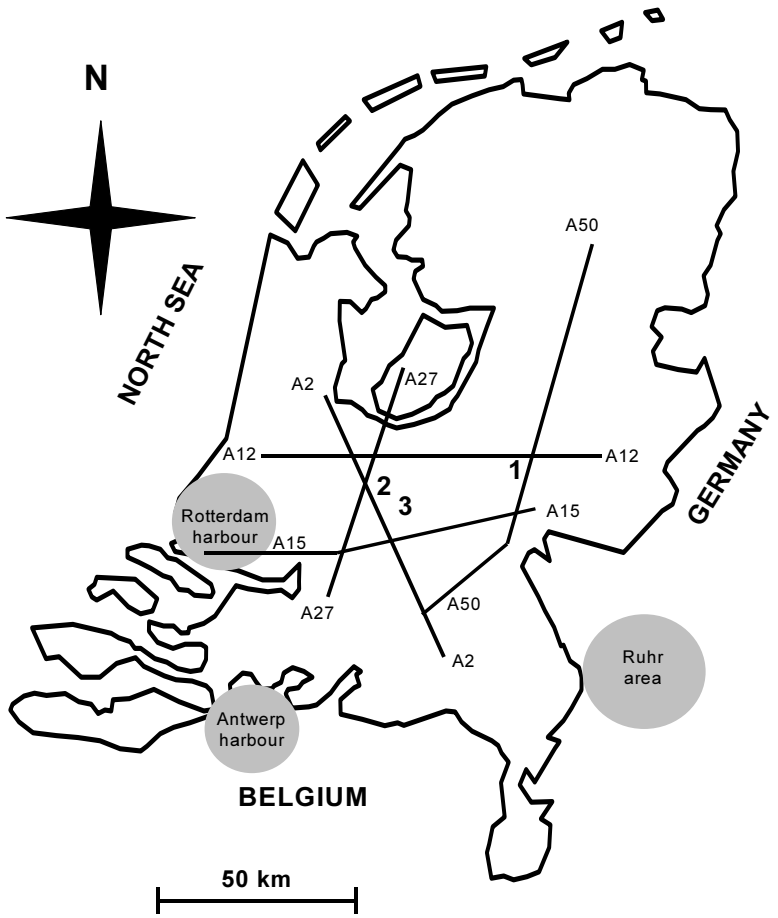


Figure 1: Sampling sites of the high-volume samplers on a schematic map of The Netherlands: 1: site PILOT located 10 m south of highway A12 near traffic point Grijsoord; 2: site HIGHWAY located 10 m east of highway A2 near exit Everdingen; 3: site BACKGROUND in nature conservation area De Regulieren near Culemborg, 5 km east of highway A2. Principal highways (together with code name) and industrial areas are indicated.

umu-*assay*

The *umu*-assay was performed in a 96 well microtiter plate. The protocol used was mainly based on the German DIN-protocol 38415-3 (DIN, 1996), especially with respect to the use of chemicals, solutions and growth media, and the times and temperatures of incubation and exposure. Our protocol deviated fundamentally from the DIN-protocol, as we tested a concentration series of the reference mutagens 4-NQO and 2-AA, rather than a single concentration and only two dilutions of the air samples.

Salmonella typhimurium TA1535/pSK1002 bacteria were grown in an overnight culture that was diluted 1:10 with growth medium on the next morning and further incubated. The test was performed both in the absence and in the presence of a metabolic activation system (*i.e.* Aroclor 1254 induced rat liver S9-mix; ICN Biomedicals Aurora, OH). 4-Nitroquinolin-oxid (4-NQO) and 2-aminoanthracene (2-AA) were used as directly and indirectly acting reference mutagens, respectively. 4-NQO and 2-AA stocks were made in DMSO and diluted with demineralized water to concentration series ranging from 19 to 600 and from 75 to 2400 µg/ml, respectively. Of each dilution (containing 4.5% of DMSO), 180 µl was added in triplicate to the microtiter plate.

Air sample extracts were diluted 6 times in DMSO and next 22 times in demineralized water yielding a 133 times diluted extract with 4.5% DMSO. In the microtiter plate, the 133 times diluted extract was further diluted in triplicate with a 4.5% DMSO solution in demineralized water into a concentration series with dilution factor = 2.5. To 180 µl of each concentration of extract, 20 µl of 10 times concentrated bacterial growth medium was added. In case of metabolic activation of the test solutions, this 10 times concentrated medium also contained co-factors ß-nicotinamide-adenine dinucleotide-phosphate (NADP) and glucose-6-phosphate.

After 1.5-2 h, the diluted bacteria culture was in an exponential growth phase and was ready for use. For experiments with metabolic activation, 450 µl of S9-mix was added to 15 ml of bacteria culture. Bacteria culture (70 µl) was added to each well (except for the background wells) and the microtiter plate was incubated at 37°C for 2 h under constant shaking. Next, 30 µl of incubated sample was diluted into 270 µl of fresh and pre-incubated (37°C) medium in a new microtiter plate, which was again incubated at 37°C and constant shaking. After 2 h, bacterial growth in the second plate was measured spectrophotometrically (λ=600 nm) and 30 µl out of each well was diluted into a third microtiter plate, containing 120 µl of pre-incubated (28°C) lysis buffer in each well. To measure β-galactosidase activity, 30 µl of o-nitrophenyl-β-D-galactopyranoside (ONPG; 4.5 mg/ml) was added to each well and the microtiter plate was incubated at 28°C. After 30 min, β-galactosidase activity was stopped by addition of 120 µl Na_2CO_3 (106 g/l) and the yellow-colored ONPG-metabolite o-nitrophenol was measured spectrophotometrically at λ=420 nm.

Both bacterial growth and β-galactosidase activity were corrected for background extinction, whereupon the enzymatic activity was corrected for growth and expressed as a fraction of the control activity, yielding the so-called induction factor. By interpolating the

induction factors of the extracts in the calibration curve of the reference mutagens, the *umu*-responses of the air extracts were expressed as equivalents of 4-NQO or 2-AA. Based on pilot results (Figures 2 and 3), this was performed for those concentrations of extracts yielding an induction factor significantly greater than the control (>1.3), which is not disturbed by possible cytotoxic effects (<2.3).

DR-CALUX-assay

The DR-CALUX assay was also performed in 96 well microtiter plates, by a method developed in our laboratory (Murk *et al.*, 1998). In addition, to distinguish between easily biodegradable and persistent AhR agonists, luciferase production was measured after 6 and 48 h with benzo[a]pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as reference PAH and PHAH, respectively. On the microtiter plate, cells were exposed to 250000 (6 h) and 250 times (48 h) diluted air samples at DMSO concentrations of 0.8%. When cells were exposed for 6 h, preconditioned filter-sterilized medium was used, in which H4IIE-cells were grown for 16 h. This procedure reduces the background signal induced by easily biodegradable tryptophan products formed by the production process of the growth medium.

After correction for the background signal, luciferase activity was expressed as a fraction of the control activity, yielding the induction factor. By interpolating the induction factors of the extracts in the calibration curve of the reference compounds, the DR-CALUX responses of the air extracts were expressed as equivalents of BaP or TCDD. This was performed for those concentrations of extracts yielding an induction factor within the linear part of the calibration curve (Figure 5).

IC-inhibition assay

The inhibition of intercellular communication was tested for only six APM samples in mouse hepatoma cells (Hepa1c1c7) as described by De Haan *et al.* (1994). Cells were exposed for 24 h to acetone:hexane (1:1) extracts dissolved in DMSO.

Data analysis

Bio-assay detection limits were analyzed by ANOVA followed by Dunnett's test. Non-parametric tests were applied to analyze effects of sampling site and prevalent wind direction on bio-assay results from APM samples, because toxic potencies were hardly ever normally distributed (Kolmogornov-Smirnov test). Differences in toxic potency between samples

collected on the same day at the two sampling sites were determined by Wilcoxon's test. Effects of prevalent wind direction on differences between sites were determined by Kruskal-Wallis test, followed by Tukey-type multiple comparisons using Nemenyi's test (Zar, 1996). Correlations between bio-assay responses were calculated using Spearman's test. If not stated differently, a significance level of $p<0.05$ has been adopted. All statistical calculations were performed using SPSS Software Package (Release 10.1, 2000).

Results

APM samples

At sampling sites HIGHWAY and BACKGROUND, airborne particulate matter (APM) was sampled on 11 and 23 working days in 1998 and 1999, respectively (Table 1).

Table 1: Characteristics of all APM samples from sites PILOT, HIGHWAY, and BACKGROUND. Based on the prevalent wind direction during sampling, samples are classified in three classes (A-C) according to the compass card shown in Figure 4.

Date	Sampling site	Sampling time	Wind direction		Class	mg APM	m^3	µg APM/m^3
		(h)	start	finish				
19-11-97	PILOT	24	SE	SE	C	52	1744.8	30
	BACKGROUND	24	SE	SSE	C	34	1785.2	19
29-04-98	HIGHWAY	24	E	NE	C	36	1483.6	24
	BACKGROUND	24	E	NE	C	38	1443.6	26
11-05-98	HIGHWAY	24	E	E	C	126	1483.6	85
	BACKGROUND	24	E	E	C	105	1279.2	82
12-05-98	HIGHWAY	24	E	NE	C	116	1398.6[a]	83
	BACKGROUND	24	E	E	C	111	1262.4	88
13-05-98	HIGHWAY	24	NE	NE	A	121	1487.2[a]	82
	BACKGROUND	24	N	E	A	90	1312.2	68
16-05-98	HIGHWAY	24	ENE	NE	C	56	1527.4	37
	BACKGROUND	24	E	E	C	65	1628.3	40
18-05-98	HIGHWAY	23.5	NE	N	A	44	1509.6	29
	BACKGROUND	24	E	N	A	49	1305.3	37
19-05-98	HIGHWAY	24	N	N	A	68	1483.6	46
	BACKGROUND	24	N	N	A	59	1305.1[a]	45
25-05-98	HIGHWAY	24	W	W	B	106	1462.6	72
	BACKGROUND	24	W	SW	B	63	1290.5	49
28-05-98	HIGHWAY	24	E	WNW	A	76	1483.6	51
	BACKGROUND	24	E	W	A	43	1307.4	33
22-06-98	HIGHWAY	44.5	W	SW	B	228	2255.4[a]	100
	BACKGROUND	48.5	W	SW	B	109	2401.4[a]	45
02-07-98	HIGHWAY	24	N	NW	A	30	1516.2[a]	20
	BACKGROUND	24	N	W	A	30	1418.7[a]	21
28-04-99	HIGHWAY	24	NNE	ENE	A	70	1604.2[a]	44
	BACKGROUND	?	N	NE	A	50	887.8	56

Date	Sampling site	Sampling time	Wind direction		Class	mg APM	m^3	µg APM/m^3
		(h)	start	finish				
03-05-99	HIGHWAY	24	NE	N	A	46	1611.2	29
	BACKGROUND	24	NNE	ENE	A	65	1536.3	43
10-05-99	HIGHWAY	24	SE	SW	B	69	1587.5	44
	BACKGROUND	24	S	SW	B	52	1536.3	34
11-05-99	HIGHWAY	24	SW	SW	B	66	1480.9	45
	BACKGROUND	24	SW	SW	B	43	1558.8	28
17-05-99	HIGHWAY	24	NEE	E	C	85	1551.8	55
	BACKGROUND	23	NE	E	C	51	1442.3 [a]	35
18-05-99	HIGHWAY	24	E	E	C	100	1553.7	64
	BACKGROUND	24	E	ENE	C	89	1541.4	58
20-05-99	HIGHWAY	24	SSW	SW	B	106	1492.2	71
	BACKGROUND	24	SSW	SW	B	75	1545.6	49
25-05-99	HIGHWAY	24	WNW	SW	B	98	1517.1	64
	BACKGROUND	24	NW	WSW	B	51	1554.7	33
26-05-99	HIGHWAY	24	SW	E	C	103	1525.7	68
	BACKGROUND	24	WSW	E	C	87	1553.4 [a]	56
27-05-99	HIGHWAY	24	E	WNW	A	128	1558.2	82
	BACKGROUND	24	E	W	A	82	1556.1	53
31-05-99	HIGHWAY	24	WNW	E	A	83	1597.1	52
	BACKGROUND	24	WNW	E	A	63	1568.9	40
07-06-99	HIGHWAY	24	ESE	WSW	C	83	1579.1 [a]	52
	BACKGROUND	47	SE	SSW	C	67	3089.8	22
09-06-99	HIGHWAY	24	WSW	W	B	76	1536.1	50
	BACKGROUND	24	WSW	WNW	B	56	1536.3	36
10-06-99	HIGHWAY	24	W	SSW	B	49	1566.7	31
	BACKGROUND	24	WNW	WSW	B	30	1577.5	19
21-06-99	HIGHWAY	24	WNW	N	A	56	1540.7	36
	BACKGROUND	24	W	N	A	13	1536.3 [a]	9
23-06-99	HIGHWAY	24	N	NW	A	70	1540.7 [a]	45
	BACKGROUND	23	N	NNW	A	76	1536.3	49
24-06-99	HIGHWAY	24	NW	NNW	A	38	1540.7 [a]	25
	BACKGROUND	24	NNW	NW	A	37	1554.6 [a]	24
12-07-99	HIGHWAY	24	NNW	W	A	75	1487.6	50
	BACKGROUND	24	N	W	A	72	1501.4	48
14-07-99	HIGHWAY	24	W	S	B	48	1501.2	32
	BACKGROUND	24	W	SSW	B	33	1527.3	22
15-07-99	HIGHWAY	24	S	S	B	51	1540.7	33
	BACKGROUND	24	SSW	SW	B	93	1593.3	58
20-07-99	HIGHWAY	24	SSE	SE	B	61	1515.0	40
	BACKGROUND	24	SW	SSW	B	41	1526.9	27
21-07-99	HIGHWAY	24	SE	S	B	51	1480.8	35
	BACKGROUND	22	SSW	SW	B	37	1424.8	26
22-07-99	HIGHWAY	24	S	NW	B	36	1527.3	24
	BACKGROUND	24	SW	WNW	B	30	1536.3	19

[a]: quartz fiber filters of these samples were slightly damaged during sampling. Due to lower resistance, the collected air preferably goes through the hole and not through the filter. Therefore, the sample volume could not be read from the gas meter. Instead, it was calculated as the annual average of sampling volumes of the intact filter samples collected with the same high-volume sampler (corrected for sampling time).

Bio-assay results from both sampling locations did not differ between samples from 1998 and 1999, so that further analysis was performed on all samples together. Based on the prevalent wind direction during sampling, samples have been divided into three groups consisting of 13, 13 and 8 sampling days (Table 1). Concentrations of APM (μg/m^3; Table 1) were significantly higher at HIGHWAY than at BACKGROUND, but APM levels per site were not affected by the prevalent wind direction during sampling.

umu-*assay*

Induction of *β*-galactosidase activity proportionally increased with concentrations of mutagens (Figure 2). For 4-NQO and 2-AA tested in the absence and the presence of S9-mix, respectively, linear dose-response curves could be calculated (linear regression). Induction factors >1.3 differed significantly from the control. Similarly, a linear dose-response relationship was found for the extracts of APM collected at sites PILOT and BACKGROUND on 19 November 1997 (Figure 3). APM extracts showed higher genotoxic potency when tested without S9-mix than with S9-mix. Besides, higher genotoxic potency was found for APM collected at site PILOT than at site BACKGROUND, with maximum induction factors of 3.0 and 2.5 (without S9-mix) and of 2.2 and 1.9 (with S9-mix), respectively. Induction factors differed significantly from the control samples (blanc filter extract) at APM extract concentrations corresponding with filtered volumes of air of ≥1.5 m^3 without S9-mix and ≥4 m^3 with S9-mix. Again, this significance corresponds with induction factors >1.3. Induction factors >2.3 no longer increased proportionally with the filtered volume of air at APM, probably due to cytotoxicity of the APM concentrations tested.

Based on these results, an optimum quantification range of induction factors was set between 1.3 and 2.3 for interpolation of APM induction factors into equivalents of reference genotoxicants. For sites PILOT and BACKGROUND, direct genotoxic potency (without S9-mix) of the samples of 19 November 1997 was equivalent to 5.7 and 3.8 ng 4-NQO/m^3, respectively, and indirect genotoxic potency (with S9-mix) to 12.8 and 7.1 ng 2-AA/m^3. Similarly, the genotoxic potency can be expressed as 191 and 201 ng 4-NQO per mg APM collected and as 430 and 376 ng 2-AA/mg, at sites PILOT and BACKGROUND respectively.

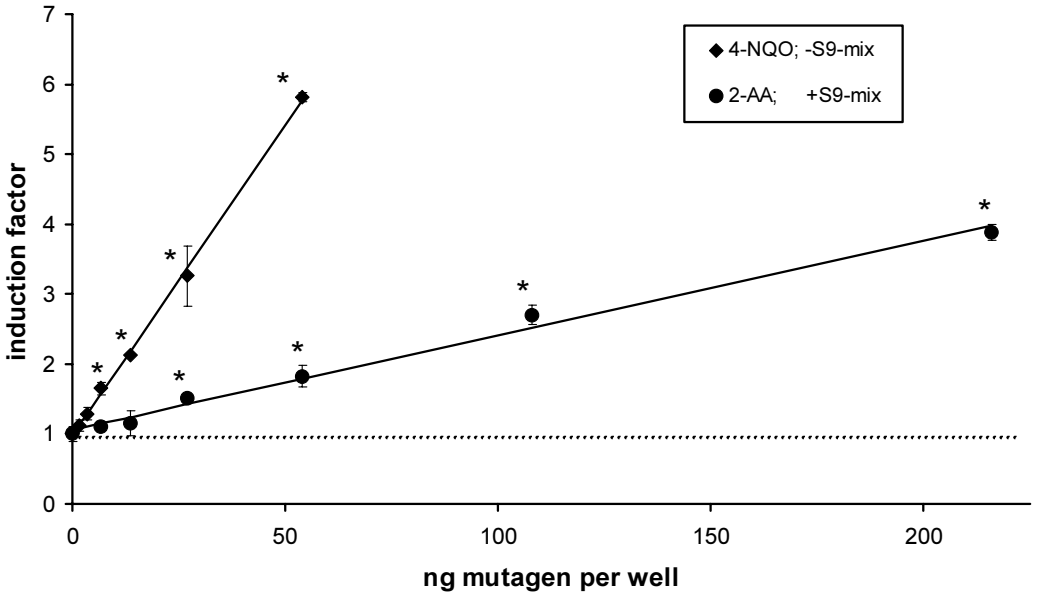


Figure 2: β-Galactosidase activity expressed as fraction of the control (with sem) found in the *umu*-assay after exposure to the reference compounds 4-NQO (without S9-mix) and 2-AA (with S9-mix). Induction factors significantly greater than the control are indicated with an asterisk.

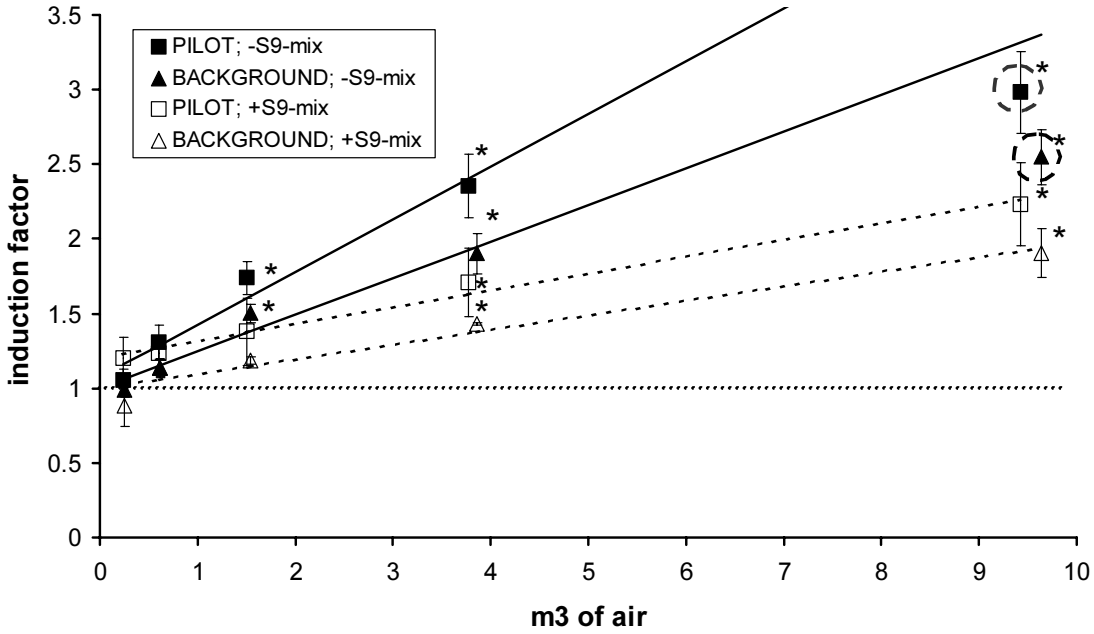


Figure 3: β-Galactosidase activity expressed as fraction of the control (with sem) found in the *umu*-assay after exposure to an air extract from 19 November 1997. Volumes on the x-axis indicate the volume (m^3) of filtered air corresponding with the extracted amount of APM, which is actually tested in each well of the microtiter plate. Squares and triangles refer to sampling sites PILOT and BACKGROUND, respectively. Closed markers with solid lines and open markers with dashed lines refer to *umu*-tests without and with S9-mix, respectively. Induction factors (IF) significantly greater than the reference level (IF=1; dotted line) are indicated with an asterisk. Encircled points are tested at cytotoxic APM concentrations and are not taken into account for the linear regression of the dose-response curves shown.

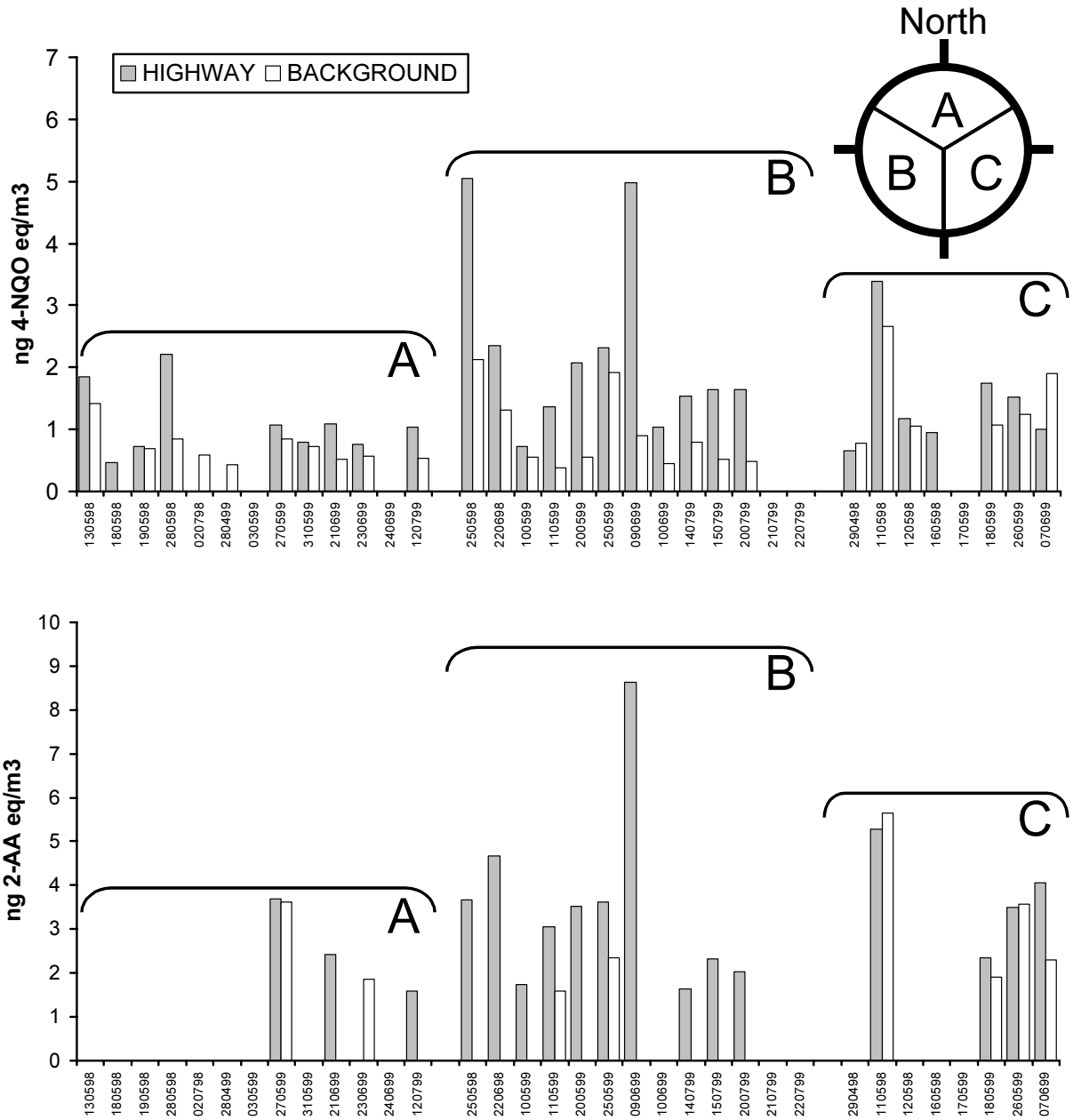


Figure 4: Average genotoxic potency without (top) and with S9-mix (bottom), recalculated into ng 4-NQO and 2-AA equivalents per m^3, respectively, of all samples collected at sites HIGHWAY and BACKGROUND from April to July in 1998 and 1999. Samples are arranged in three groups (A-C) according to the prevalent wind direction during sampling (see compass-card). Samples for which no bars are shown had induction factors <1.3 and have therefore not been recalculated into equivalent concentrations of reference compounds.

Extracts of APM collected at sites HIGHWAY and BACKGROUND between April and July in 1998 and 1999 were tested in two concentrations, both in absence and presence of S9-mix. Figure 4 shows the average direct and indirect genotoxic potency per m^3 of filtered air, with samples being arranged according to the prevalent wind direction during sampling. Both direct and indirect genotoxicity were significantly higher for APM from site HIGHWAY than

from BACKGROUND, but differences between sites were relatively small. Ranges of genotoxic potencies were equivalent to <0.15-5.1 ng 4-NQO per m^3 (without S9-mix) and to <0.8-8.6 ng 2-AA per m^3 (with S9-mix). Highest direct genotoxic potency was found for HIGHWAY samples from 25 May 1998 and 9 June 1999. At maximum, genotoxic potency of APM was 5.6 times higher at site HIGHWAY than at BACKGROUND (9 June 1999; Figure 4). Differences in genotoxic potency between sites HIGHWAY and BACKGROUND were significantly affected by the prevalent wind direction. With westerly wind, differences between sites were significantly higher than with northerly (with and without S9-mix) and easterly (only with S9-mix) wind.

Out of the 54 samples containing genotoxic potency without S9-mix, 29 were not genotoxic after metabolization with S9-mix (Figure 4). Moreover, no samples were activated by S9-mix that were not active without S9-mix. A significant correlation (ρ=0.58) was found between the remaining 25 samples showing genotoxic potency with and without S9-mix.

DR-CALUX assay

The DR-CALUX assay shows a typical sigmoid dose-response relationship when exposed to BaP for 6 h, with a maximum induction factor of 15 at $1*10^5$ pM BaP (Figure 5). As the arrows in Figure 5 indicate, $1.5*10^5$ times diluted extracts of APM collected at sites PILOT and BACKGROUND on 19 November 1997 generated a DR-CALUX response within the linear part of the calibration curve. Induction factors of 6.3 and 5.6 were interpolated into BaP-equivalent concentrations of 1060 and 793 pM, respectively. For PILOT and BACKGROUND, these equivalent concentrations were recalculated into 9.1 and 6.5 pmol BaP/m^3 of sampled air and into 305 and 342 pmol BaP/mg APM collected, respectively. TCDD-equivalents determined in the DR-CALUX assay with 48 h of exposure were 2.6 and 1.4 fmol TCDD/m^3 (*i.e.* 87 and 74 fmol/mg) for sites PILOT and BACKGROUND, respectively.

Similar to the *umu*-assay results, responses of the DR-CALUX assay after 6 h of exposure to extracts from HIGHWAY samples were significantly higher than from BACKGROUND samples (Figure 6), with BaP-equivalent concentrations ranging from 2.7 to 79.0 and from 3.4 to 23.8 pmol/m^3 air, respectively. Differences between HIGHWAY and BACKGROUND samples were larger with westerly (B) than with northerly (A) wind (p=0.054), with a maximum ratio between sites (factor 9.5) on 20 May 1999. A significant correlation (ρ=0.59) was found between DR-CALUX responses after 6 h of exposure and *umu* responses in absence of S9-mix.

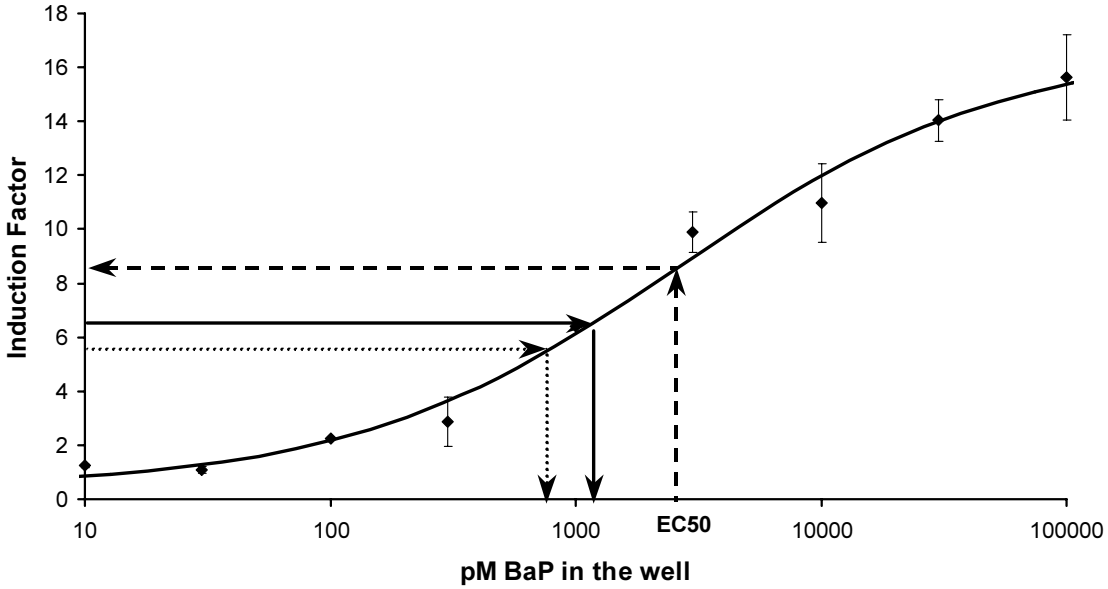


Figure 5: Dose-response related DR-CALUX response after 6 h of exposure to benzo[a]pyrene. Induction factor is light production in the well expressed as a fraction of the control. Solid and dotted arrows indicate the interpolation of the response of the 150000 times diluted samples from sites PILOT and BACKGROUND, respectively, on 19 November 1997. The dashed arrow indicates the calculated EC_{50}-value (=2.6 nM BaP).

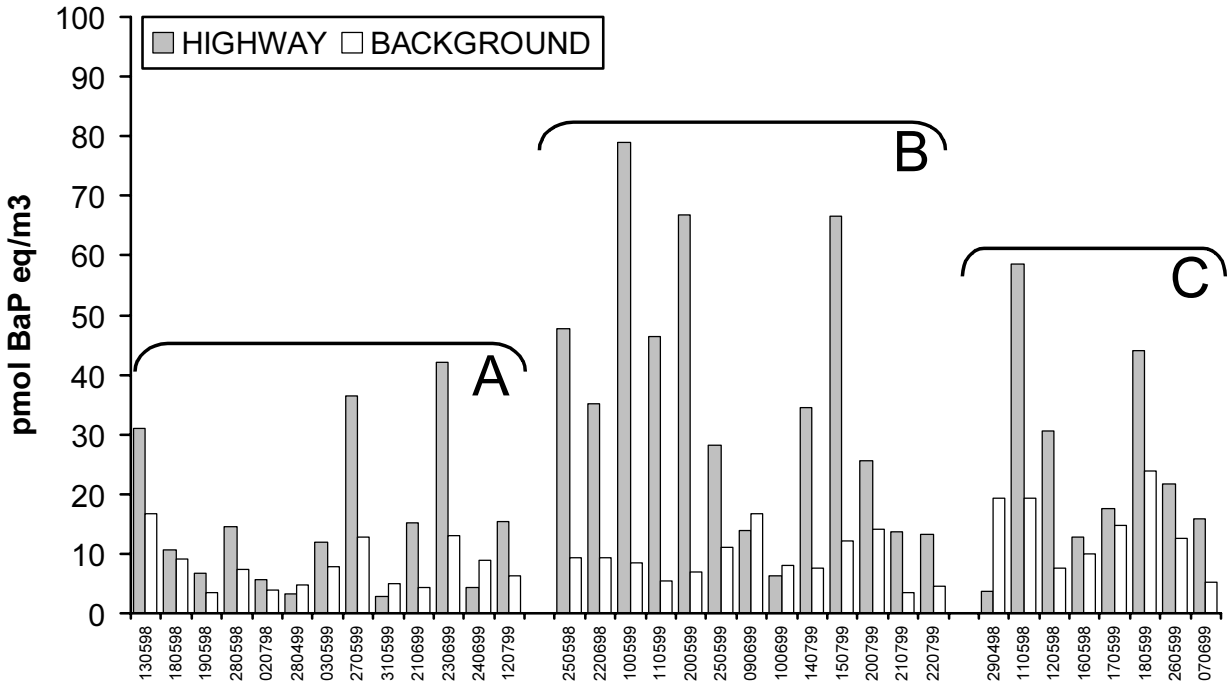


Figure 6: AhR activating potency recalculated into pmol BaP-equivalents per m^3 after 6 h of exposure to APM collected at sites HIGHWAY and BACKGROUND between the end of April and the beginning of July in 1998 and 1999. Samples are arranged in three groups (A-C) as in Figure 4.

No DR-CALUX response was found after 48 h of exposure to APM extracts from 1998 and 1999, implying that the airborne toxic potency of stable AhR agonists was <1 fmol TCDD equivalents per m^3.

Intercellular communication

In tissue cultures, intercellular communication between hepatoma cells was inhibited after 24 h of exposure to extracts of 10 m^3 of air collected at sites PILOT and BACKGROUND on 3 different days. Levels of communication between exposed cells ranged from 45 to 63% of the control level, indicating the presence of tumor promoting activity in the APM samples. For the few samples tested, the response seemed to be independent from sampling sites and from the prevalent wind directions during sampling.

Discussion

Obviously, the selected reporter gene assays are applicable for testing the toxic potency of APM, as the results demonstrate the presence of both genotoxic and PAH-like compounds in the complex mixture of diffuse air pollution. Bio-assay responses from sites HIGHWAY and BACKGROUND were always within one order of magnitude, indicating that airborne pollutants with genotoxic and/or AhR activating potency are widely dispersed throughout the atmosphere. This omnipresent character of diffuse air pollution confirms that no negative control sites are available to characterize the toxic potency of the gray veil of air pollution in The Netherlands, and that background levels of pollution should be compared to a worst case situation instead.

With westerly wind (class B), both *umu* and DR-CALUX assay indicated large differences in toxic potency between APM from sites HIGHWAY and BACKGROUND. This result is explained by the fact that the high volume sampler was situated east of the road at site HIGHWAY, so that APM was collected down-wind from the highway during conditions of westerly wind. With northerly (marine) wind (class A), least toxic potency was found in both bio-assays, with small differences between sampling sites. This is in agreement with earlier results by Van Houdt *et al.* (1987), who found relatively low mutagenic activity in the Ames assay in samples of APM originating north from The Netherlands. Still, with northerly wind genotoxic and AhR agonistic potency were found in 77% and 100% of the APM samples from the BACKGROUND site, indicating that a certain basic level of pollution is present under

all sampling conditions and that differences between sites are related to additional local emissions. With easterly wind conditions (class C), large variation was found between sampling days. However, differences between sites were small, even on relatively polluted days (*e.g.* 110598 and 180599), suggesting that local emission of air pollutants was less important on these days than long-range transport. APM samples classified as class C often also had a different composition, given the relatively high response in the *umu*-assay with S9-mix for samples from site BACKGROUND.

Apparently, the prevalent wind direction during sampling greatly affects the toxic potency of APM on both sampling sites. Nevertheless, some samples disturb the general idea that APM from northerly wind is clean, from westerly wind is highly dominated by highway emissions and from easterly wind incidentally contains (imported) pollutants. For instance, relatively high bio-assay responses were found for the northerly samples of 130598, 280598 and 270599, probably because these samples were not correctly classified based on the wind direction recorded at the start and the end of each sampling period. These errors may be due to the fact that samples were collected while wind directions were changing (*e.g.* 280598) or while wind directions were exactly on the verge between two wind classes (*e.g.* 130598) (Table 1). More detailed information on the origin of the air package sampled was obtained by so-called trajectory calculations, which were performed by the Royal Netherlands Meteorological Institute (KNMI) for 6 sampling days in 1998. Figure 7 shows the route (trajectory) of a virtual APM particle that passed the high-volume sampler about 8 meters above ground level at the start (t=0), halfway (t=12) and at the end (t=24) of each sampling period.

Indeed, samples from 130598 are mainly characterized by easterly wind directions and should therefore have been classified as class C rather than class A. More interesting are samples from 280598, which are characterized by APM from southerly directions at the start, from easterly directions halfway, and from westerly directions at the end of the sampling period (Figure 7). Although it is likely that wind turned over north on 280598, it is actually impossible to classify these samples in one of the classes defined. Although these examples clearly illustrate the usefulness of trajectory analyses in explaining the toxic potency of APM samples, the original classification based on ground-level measurements of wind directions at start and end of each sampling period has been adopted in this paper, because they were available for all samples and were collected in a standardized way. Besides, they sufficiently described the origin of APM for most samples, as demonstrated for the samples from 120598,

190598 and 250598 (Figure 7; right column), which were correctly classified as class C, A and B, respectively (Table 1).

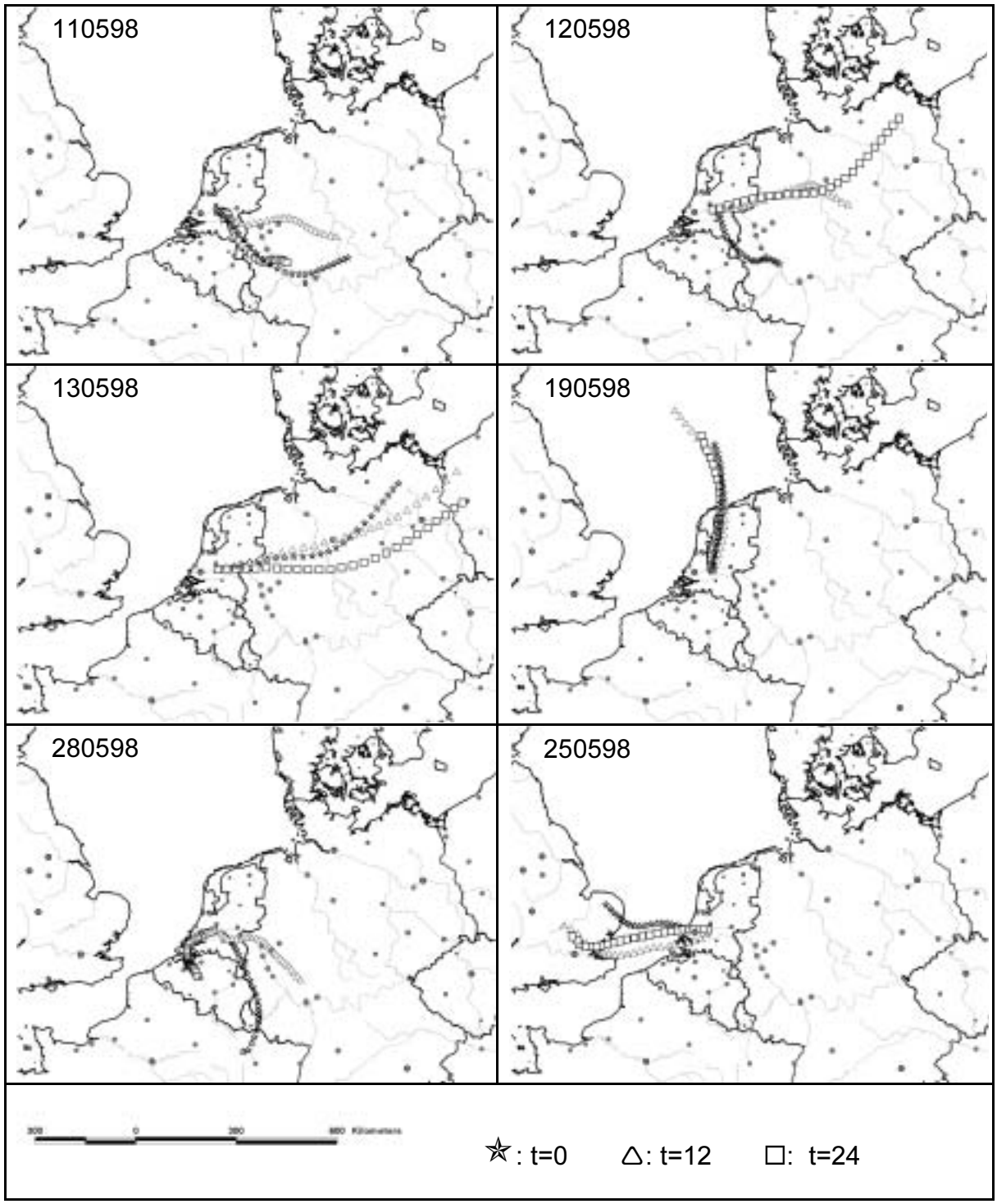


Figure 7: Trajectory projections of air masses sampled on 6 different sampling days. For each sampling day the route is projected of a virtual particle that passed the high volume sampler about 8 meters above ground level at the start (t=0), halfway (t=12) and at the end (t=24) of each sampling period. Each trajectory route is represented by 24 different points indicating the position of the particle 1 to 24 h before passing the high volume sampler.

Trajectory analysis also indicated that highly genotoxic APM sampled on 110598 came from a package of air originating from the industrial Ruhr area in Germany (Figure 7). Such transboundary long-range transport of mutagens has previously been described by Van Houdt *et al.* (1987), and is supported by the small differences in *umu*-responses between sampling sites on 110598, so-that local sources can be excluded. Besides, the samples had a genotoxic profile deviating from most other samples consisting of many S9-persistent or S9-activated compounds.

For all APM samples tested, genotoxicity without S9-mix was higher than with S9-mix. Possibly, direct genotoxic compounds present in APM extracts are metabolized by S9-mix into non-genotoxic compounds, so that the *umu*-response in presence of S9-mix is actually caused by direct mutagenic compounds, which have not been metabolized yet by the rat liver homogenate. In that case, increased genotoxicity at site HIGHWAY with westerly wind may be attributed to (di-)nitro-PAHs. Especially (di-)nitropyrenes are known to be side-products of diesel combustion: 1-nitropyrene and 1,8-dinitropyrene are expected to be totally responsible for 20 and 80%, respectively, of the direct mutagenicity of diesel exhausts (Rosenkranz, 1982). Furthermore, these nitropyrenes seem to be resistant to breakdown by S9-mix: Scheepers *et al.* (1995) reported high correlation between both direct and indirect mutagenicity and 1-nitropyrene contents of air sample extracts, with less mutagenic activity in presence of S9-mix. The persistence of these compounds to S9-mix may explain the relatively high genotoxic activity with S9-mix found for many HIGHWAY samples collected with westerly wind (class B) in the present study (Figure 4). Higher genotoxic potency without than with S9-mix can also be explained by the fact that active compounds in the extract bind to proteins of the S9-mix and become less available for *S. typhimurium*-bacteria, as was suggested by additional experiments with S9-mix inactivated by heat (data not shown). Finally, direct genotoxicity may also be attributed to highly reactive oxidized PAHs (including nitropyrene), which can be formed by photomodification of traffic exhausts and have a higher mutagenic potency than their parent PAH compounds (Huang *et al.*, 2002).

The correlation between *umu* responses (without S9-mix) and DR-CALUX (6 h of exposure) suggests that APM contains compounds with both genotoxic and AhR agonistic characteristics, such as nitro-PAHs. On the other hand, PAHs make up only a part of the wide variety of compounds that both bio-assays respond to. Therefore, the observed correlation may as well be explained by the fact that both assays cover two broad spectra of airborne

pollutants of which the concentrations correlate in general, *i.e.* high concentrations during polluted days and low concentrations during clean days.

DR-CALUX responses after 6 h of exposure have been expressed in equivalents of benzo[a]pyrene, which is a readily biodegradable PAH. In theory, both biodegradable and persistent AhR agonists induce the 6 h DR-CALUX response, whereas only persistent compounds induce the response after 48 h. Because TCDD-equivalents (48 h) were <1 fmol/m^3 in the 1998 and 1999 APM samples of HIGHWAY and BACKGROUND, it is concluded that the contribution of persistent AhR agonists as dioxin-like compounds is negligible compared to the contribution of more easily biodegradable agonists such as PAHs. Possibly, the low levels of PHAHs in the air should be attributed to recent, effective administrative actions in The Netherlands to reduce emissions of especially dioxins by a ban on leaded gasoline and by refurbishment of waste incinerators.

Pilot experiments with extracts from APM collected at sites PILOT and BACKGROUND on three different days, showed an IC-inhibition ranging from 37 to 55% after 24 h of exposure to extracts of 10m^3 of air. Heussen (1991) previously described IC-inhibiting capacity of extracts from outdoor APM, which correlated with its mutagenic potency.

When responses from *umu* and DR-CALUX assays were expressed as equivalent concentrations per mg of APM sampled instead of per m^3 of air filtered, differences between sampling sites were still significant but less pronounced, probably due to the fact that APM-levels themselves were significantly higher at site HIGHWAY than at BACKGROUND (µg APM/m^3; Table 1). Moreover, extra uncertainty is introduced by expressing toxic potencies per mg APM, because some quartz fiber filters were damaged during sampling, so that quartz fiber material was ripped from the filter. Thus, the APM-weight that was determined as the difference in filter weight after and before sampling is underestimated and its toxic potency is overestimated. Filter damage led to less uncertainty in toxic potency expressed per m^3 of air, because sound assumptions could be made on the volume of air sampled based on average sampling volumes of the high-volume samplers. Although reduced filter solidity obviously is a drawback, pre-washing of quartz filters is necessary. In contrast to glass fiber filters, which contain high levels of heavy metals, cytotoxicity of quartz filters was negligible after washing.

As described in Material and Methods, the *umu*-protocol used was mainly based on the German DIN-protocol (DIN, 1996), but deviated from this protocol, as we tested a concentration series of reference compounds 4-NQO and 2-AA enabling calculation of the

dose-response calibration curves of the reference mutagens. Interpolation of *umu*-responses from single concentrations of APM extracts in the calibration curves of the reference mutagens, enabled us to express the genotoxic potency of the extracts as equivalent concentrations of reference mutagens. This yields an absolute, quantifiable measure instead of a relative measure such as "the smallest dilution of the sample yielding an induction factor >1.5", which is the reported measure according to the DIN-protocol. An additional advantage is that there is no longer a need of testing multiple concentrations of APM extracts and that the capacity of the assay increases (*i.e.* the number of samples tested per microtiter plate).

Of course, the selected bio-assays only cover a specific part of all the chemicals present in the complex mixture of air pollution. Future research will focus on selecting new bio-assays for testing the toxic potency of other airborne pollutants. These assays will meet the same criteria as the assays used in the current study, *i.e.* being able to identify and quantify relevant toxic endpoints, and to respond specifically to groups of chemicals known or suspected to be predominant constituents of polluted air or deposition.

Conclusions

The selected reporter gene assays demonstrate the presence of pollutants with genotoxic and/or PAH-like characteristics in the complex mixture of airborne particulate matter. This diffuse air pollution forms an undesired mutagenic risk of which the extent depends on location and on the prevalent wind direction during sampling. However, an omnipresent basic toxic potency can be detected even under the cleanest circumstances studied, *i.e.* in a background area under conditions of northerly (marine) wind. This background toxic potency of APM is only less than one order of magnitude smaller than under relatively polluted circumstances, as regularly found down-wind from a highway and incidentally under conditions of easterly wind. The increased toxic potency related to easterly wind directions is attributed to long distance transport of airborne pollutants, because it is independent from sampling location and has a different composition than traffic related pollution. Trajectory analyses suggest that such polluted air packages descend from the industrial Ruhr area in Germany, and proved to be valuable tools to determine the origin of APM pollutants and to explain bio-assay results from samples collected under changing wind directions. Dioxin-like activity of APM is <1 fmol TCDD equivalents per m^3 of air, and is concluded to be

negligible. Future research on diffuse air pollution will focus on further evaluation of the mutagenic risk *in vivo*.

Acknowledgements

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Chapter 3

A small-volume bio-assay for quantification of the esterase inhibiting potency of mixtures of organophosphate and carbamate insecticides in rainwater: development and optimization

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Timo Hamers
Kim RJ Molin
Jan H Koeman
Albertinka J Murk

Open rainwater samplers in nature conservation area De Regulieren (left), and amber bottles containing 14-day rainwater samples ready for transport to the laboratory (right). The jerry can contains demineralized water to wash the sampling funnels in the field.

Abstract

The goal of this study was to develop a sensitive *in vitro* bio-assay for quantification of the total esterase inhibiting potency of low concentrations of organophosphate and carbamate insecticides in relatively small rainwater samples. Purified acetylcholinesterase (AChE) from electric eel (*Electrophorus electricus*) and carboxylesterases from a homogenate of honeybee heads (*Apis mellifera*) were used as esterases, each having different affinities for the substrates S-acetylthiocholine-iodide (ATC) and N-methyl-indoxylacetate (MIA). MIA-hydrolysis by honeybee homogenate was more sensitive to inhibition by organophosphate insecticides than ATC-hydrolysis by purified AChE, although the latter parameter is often used in *in vitro* monitoring of esterase inhibitors. The higher sensitivity of carboxylesterases is attributed to the instant formation of a reversible Michaelis-Menten complex with the inhibitor, which competes with MIA for the active sites of the free enzymes. This dose-dependent instant inhibition can be quantified with kinetics for competitive inhibition at dichlorvos concentrations <16 nM. At similar concentrations, AChE was not instantly inhibited, whereas both AChE and carboxylesterases were irreversibly and progressively inhibited at higher dichlorvos concentrations ($IC50_{10min} \geq 0.1$ μM). Honeybee homogenate mediated MIA hydrolysis was applied as the most sensitive enzyme-substrate combination for experiments with fractionated extracts of 4 rainwater samples collected in a natural conservation area. Most esterase inhibiting potency was found in the polar methanol fraction, with recalculated concentrations equivalent to 12-125 ng dichlorvos per liter rainwater.

Introduction

The present study was carried out in order to optimize a methodology for the assessment of complex mixtures of esterase inhibiting insecticides, which are known to be an important group of air polluting compounds in The Netherlands. Together with neighboring country Belgium, The Netherlands uses relatively the most pesticides per ha of arable land of all EU-countries (Brouwer and Van Berkum, 1998; RIVM/CBS, 1999). Despite measures taken to reduce pesticide use, Dutch emissions to the air compartment in 1995 were still estimated to be 3110 tons of active ingredient, which is more than 95% of the total environmental loading with pesticides (Draaijers *et al.*, 1998). Pesticide emissions to the air are mainly due to volatilization from soil surfaces and plants, and further to spray drift and diffusion. Airborne

pollution can easily reach relatively protected areas, especially in a densely populated country as The Netherlands where these areas are always close to sources of pollution. In 1998, esterase-inhibitors such as organophosphate and carbamate insecticides made up almost 70% of the total amount of insecticides sold in The Netherlands (RIVM/CBS, 1999). From an (eco-)toxicological point of view, these compounds form an important part of the complex mixture of airborne pesticides, because they are toxic to a broad spectrum of species and are efficiently removed from the atmosphere by wet deposition, due to their relatively polar character. In the Netherlands, the presence of organophosphate and carbamate compounds in rainwater has clearly been demonstrated by several research groups, often in concentrations exceeding the Dutch quality criteria for surface water (Fleverwaard, 1993; Province of South-Holland, 1994; Baas and Duyser, 1997). For example, maximum concentrations of dichlorvos in the three studies ranged from 0.4-4.5 µg/l.

In order to carry out a hazard assessment of the complex mixture of pesticides in rainwater, knowledge of the concentration and the consequent toxicity of an individual pesticide are insufficient, because combination effects such as synergism or antagonism are neglected. Furthermore, unexpected toxic pesticides or metabolites of pesticides are missed when only the parent compounds expected to be present are analyzed. Therefore, a better approach could be to measure the integrated toxic potency of the complex mixture using a bio-assay that enables identification and quantification of relevant toxic endpoints. The toxicity of organophosphate and carbamate insecticides is based on their ability to block esterases, thus inhibiting the hydrolysis the neurotransmitter acetylcholine by acetylcholinesterase (AChE). Since the end of the 1940s, esterase inhibition has been measured for the monitoring of organophosphate compounds (O'Brien, 1960), by manometric (Metcalf and March, 1949) and spectrophotometric (Hestrin, 1949) techniques. Esterase activity was often determined in mammalian erythrocytes or in crude homogenates made from insects or vertebrate tissues after *in vivo* or *in vitro* exposure to inhibitors. Sensitivity, applicability and simplicity were considerably improved by the introduction of a new colorimetric technique using acetylthiocholine as a specific substrate for AChE (Ellman *et al.*, 1961). Brogdon and Barber (1987) successfully downscaled this method to a microtiter plate assay, allowing quantification in the order of 0.1-10 µM.

The objective of the current study was to develop a more sensitive small-volume assay for measuring esterase inhibition, enabling quantification at lower concentrations of inhibitors. To select the optimum test conditions, inhibition kinetics were studied for the hydrolysis of

two substrates (*i.e.* specific S-acetylthiocholine-iodide or non-specific N-methylindoxylacetate) by two types of esterases, *i.e.* purified AChE from electric eel (*Electrophorus electricus*), which is commercially available, and a homogenate of honeybee heads (*Apis mellifera*). The latter type of esterases was chosen because honeybees are known for long to be sensitive to organophosphate and carbamate insecticides (*e.g.* Anderson and Atkins, 1966; Anderson *et al.*, 1968; Atkins *et al.*, 1970a; Atkins *et al.*, 1970b; Johansen, 1969). Dichlorvos was used as a model organophosphate inhibitor allowing quantification of the mixture toxicity of the rainwater extract into dichlorvos equivalent concentrations. SPE-extraction of rainwater was optimized using methanol, dichloromethane and hexane for subsequent elution of extracted pesticides.

Materials and Methods

Chemicals

Fresh stock solutions of 28.9 g/l (100 mM) S-acetylthiocholine-iodide (ATC; Merck) were made weekly in demineralized water at kept 4°C in the dark. Dithiobisnitrobenzoic acid (DTNB [Ellman's reagent]; Fluka) was used as an indicator for ATC-hydrolysis. Fresh stock solutions were made monthly in P-buffer (0.1 M KH_2PO_4/K_2HPO_4; pH=7) and kept at 4°C in the dark. Fresh stock solutions of 1 mg/ml (5.3 mM) N-methylindoxylacetate (MIA) (1-H-indol-3-ol, 1 methyl-acetate [ester]; Sigma) were made weekly in methanol and kept at -20°C in the dark. Dichlorvos (2,2-dichloroethenyl-dimethyl-phosphate [CAS 62-73-7]; Mw=220.98; Riedel-De Haën) was used as a standard organophosphate insecticide. Pesticide mix contained 200 µg/ml of 20 organophosphate compounds in hexane (azinphos-methyl [86-50-0], chlorpyrifos [2921-88-2], coumaphos [56-72-4], demeton [8000-97-3], diazinon [333-41-5], dichlorvos [62-73-7], disulfoton [298-04-4], ethoprophos [13194-48-4], fenchlophos [299-84-3], fensulfothion [115-90-2], fenthion [55-38-9], merphos [150-50-5], mevinphos [7786-34-7], naled [300-76-5], parathion-methyl [298-00-0], phorate [298-02-2], prothiofos [34643-46-4], sulprofos [35400-43-2], tetrachlorvinphos [961-11-5], and trichloronat [327-98-0]) and was obtained from Baker. Both stocks were diluted in methanol and kept at 4°C in the dark. On each experimental day, fresh concentration series of pesticide standards were made in P-buffer. Stock solutions of 5 U/ml pure AChE (electric eel; Sigma) in P-buffer were made freshly on each test day.

Homogenate of honeybee heads

Worker honeybees (*Apis mellifera*) were obtained from the Laboratory of Entomology (Wageningen University). Bees were anaesthetized with CO_2, frozen to death at -20°C and decapitated. With a potter instrument, 18 heads were six times homogenized in 2 ml of P-buffer. After each homogenization step, the potter tube was put on ice, allowing the chitinous rests of the crushed bee heads to sink down. Next, the overstanding crude homogenate was collected and 2 ml of fresh P-buffer was added to the deposited rest of the bee heads and homogenization was repeated. After the last step all crude homogenate collected (about 12 ml) was centrifuged (5 min; 4388 g). The supernatant was divided into small batches (250 and 500 µl) and frozen at -20°C. Protein levels were measured by the method of Bradford (1976) and ranged from 1.3 to 1.5 g/l.

Esterase inhibition assay

All experiments were performed in P-buffer in a microtiter plate. ATC was used as a substrate to determine specific cholinesterase activity. ATC hydrolysis was measured as an increase in OD_{412} (15 s time intervals; SpectraMax 340 platereader [Molecular Devices Corporation]) caused by the reaction of thiocholine with DTNB to produce the yellow 5-thio-2-nitro-benzoic acid anion (Ellman *et al.*, 1961). MIA was used as a substrate to determine non-specific esterase activity. MIA hydrolysis was measured as an increase in fluorescence (70 s time intervals; CytoFluor™ 2350 platereader [Millipore]) caused by the hydrolysis product N-methyl indoxyl (λ_{ex}=418 nm; λ_{em}=500 nm).

In all microtiter plates, one pesticide or sample was tested in several concentrations. First P-buffer was added to reach final volumes of 300 and 200 µl in all wells using ATC and MIA as a substrate, respectively. Next, 50 µl of inhibitor was added and incubation was started at time t_0 by adding 50 µl of enzyme solution. To correct for non-enzymatic hydrolysis, some wells received P-buffer instead of enzyme. With ATC as a substrate, enzyme concentrations added were 75 µl bee homogenate per ml. With MIA, concentrations added were 15.2 µl bee homogenate per ml or 102 mU eel AChE concentrations per ml.

Two different protocols have been used. In protocol I experiments, one single concentration of substrate was added to the different columns of the microtiter plate after 4 different incubation periods t_1-t_4, enabling following of progressing irreversible inhibition. In protocol II experiments, 4 different substrate concentrations were added to the different

columns of the microtiter plate immediately after addition of the inhibitor, enabling measurement of instant reversible inhibition. Treatments were measured in triplicate.

Applying protocol I with ATC as a substrate, 100 µl of DTNB (5mM in P-buffer) and 100 µl of ATC (0.795 mM in P-buffer) were added at time points t_1-t_4 and with MIA as a substrate, 10 µl of MIA (5.3 mM in methanol) was added. Increases in absorption and fluorescence, respectively, were measured immediately after substrate addition.

Using ATC as a substrate in protocol II, 100 µl of DTNB and 100 µl of 0.143, 0.239, 0.398 and 0.795 mM ATC in P-buffer were added immediately after incubation had started and increase in absorbance was measured. Using MIA as a substrate in protocol II, 10 µl of 0.954, 1.59, 2.65 and 5.3 mM MIA in methanol were added immediately after incubation had started and fluorescence was measured.

Rainwater sampling

Rainwater was sampled in a natural conservation area (De Regulieren near Culemborg), which is considered to be a relative background area for the Dutch situation. Rainwater samples were collected at 1.5 m above ground level in 10 open samplers. Each sampler consisted of a clean amber glass bottle (2.5 l), with a polyester funnel (∅=24 cm) on top. Samples were collected within 2 days after major showers: April 29, May 7, May 16, and June 13, 1997. By then, bottles had remained in the field for a period of 6, 8, 9 and 23 days, respectively.

Sample processing

At arrival at the laboratory, pH was measured in each bottle and samples were stored in the dark at 4°C. Out of the total sample, a 6 l sub-sample was filtered over a glass fiber filter (Schleicher & Schuell GF50; ∅=47mm). Filters were replaced when clogged. On the average, 2-4 filters were needed to filter the sub-sample. Filters were dried and pH of the sub-sample was set at 5.0 (± 0.1) with 1N HCl or NaOH. Solid phase extraction was performed using an extraction disk, made of rigid glass fiber material with polystyrene divinylbenzene as active phase (Ztek Accu.Flo™ SDVB Fiber SPE Disks). The disk was pre-washed with methanol, dichloromethane and hexane (3*10 ml per solvent). After drying, the disk was conditioned with 10 ml of methanol for 15 min after which 300 ml of demineralized water was pulled through the disk to wash away the methanol using a vacuum pump. Finally, the sub-sample of 6 l was pulled through the disk. After extraction, the 2-4 glass fiber filters were put upon the

dried disk and both disk and filters were eluted successively with methanol, dichloromethane and hexane (three times 2 ml per solvent). Per solvent, each extract was collected separately, evaporated under a gentle nitrogen stream at 37°C, and dissolved in 10 µl of 1-propanol as carrier. Extracts were kept in the dark at 4°C and were freshly diluted into 2 ml of P-buffer (4°C) on each experimental day. Next, the diluted extract was further diluted into a linear concentration series, with dilution factors depending on the esterase inhibiting potency of the sample and with each dilution having a final volume of 650 µl, allowing to add 12*50 µl to the microtiter plate. For each sample, 1-propanol concentrations were standardized, with a maximum final concentration of 0.07% in the microtiter plate.

Spike analysis for recovery assessment

A batch of 6 l demineralized water was spiked with the pesticide mixture (100 µg of each constituent). The spiked sample and a control sample of 6 l demineralized water were extracted similar to the rainwater samples. The pesticide mix was chosen to represent a general mixture of OPs that may be present in rainwater, containing not only oxon-forms, but also less potent thio-forms. Recovery was calculated by comparing the esterase inhibiting potency (protocol II) of the 100-fold diluted extract of the spiked batch (final volume = 1 ml) to the potency of a 100 µg/ml solution prepared directly from the pesticide mixture itself.

Data analysis and statistics

In the present study, special emphasis was given to express the overall toxic potency of a mixture of compounds into a reliable single value, which is easy to interpret. Esterase inhibiting potencies were recalculated into equivalent concentrations of dichlorvos (with 95% confidence interval) based on the reaction kinetics of esterase mediated hydrolysis. Both substrates (such as ATC and MIA) and inhibitors (such as organophosphate and carbamate insecticides) are hydrolyzed by esterases according to the same ubiquitous reaction scheme (Main, 1964; Aldridge and Reinier 1972):

$$EH + AB \underset{k-1}{\overset{k+1}{\rightleftarrows}} EHAB \xrightarrow{k+2} EA + BH \xrightarrow[+H_2O]{k+3} EH + AOH + BH \qquad (1).$$

First, the substrate or inhibitor AB quickly binds to the free esterase EH to form a reversible Michaelis-Menten complex (EHAB). Next, the enzyme is irreversibly carboxylated (in case of a substrate as MIA or ATC) or phosphorylated or carbamylated (in case of an inhibitor as organophosphates or carbamates, respectively) into EA, and BH is split off

simultaneously. Finally, EA is hydrolyzed into EH and AOH. As the rate of hydrolysis (k_{+3}) is much slower for inhibitors than for substrates, they inhibit esterases irreversibly.

In view of the results, most emphasis was given to the quantification of the reversible inhibition of honeybee homogenate mediated MIA-hydrolysis by small concentrations of OP-esters, which could be described by Michaelis-Menten kinetics for competitive inhibition:

$$v_0 = \frac{V_{\max} * [S]}{K_m * \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (2)$$

with v_0 is the initial rate of the enzyme catalyzed reaction immediately after addition of the substrate to the enzyme, V_{max} is the maximum initial reaction rate, K_M is the Michaelis constant, K_i is the inhibitor constant, [S] is the substrate concentration, and [I] is the inhibitor concentration. K_M and K_i are both constants which can be interpreted as typical concentrations: K_M is the substrate concentration at which the initial reaction rate (v_0) in the absence of an inhibitor ([I]=0) is equal to $½V_{max}$ and K_i is the inhibitor concentration for which twice as much substrate is needed to reach an initial reaction rate (v_0) of $½V_{max}$ than in an uninhibited situation. Thus, esterase-inhibiting capacity increases with decreasing K_i-values.

To recalculate the esterase inhibiting potency of a sample into equivalents of a standard organophosphate insecticide (*i.e.* dichlorvos), K_i-values of both samples and dichlorvos were calculated by performing a reciprocal transformation of the Michaelis equation (2) into the Lineweaver-Burk equation

$$\frac{1}{v_0} = \frac{K_M}{V_{\max}} * \left(1 + \frac{[I]}{K_i}\right) * \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (3).$$

This again was re-written into

$$\frac{1}{v_0} = \frac{K_M}{V_{\max}} * \frac{1}{[S]} + \frac{K_m}{V_{\max} * K_i} * \frac{[I]}{[S]} + \frac{1}{V_{\max}} = p * \frac{1}{[S]} + q * \frac{[I]}{[S]} + r \quad (4).$$

Multiple linear regression was performed on 1/[S] and [I]/[S], with least squares being weighed by $(v_0)^2$ (SPSS for Windows, version 7.5.2). K_i was estimated by dividing the regression coefficients:

$$K_i = \frac{p}{q} \quad (5).$$

A 95% confidence interval for K_i was calculated applying Fieller's theorem (Fieller, 1954; Collett, 1994):

$$L = \frac{-b \pm \sqrt{b^2 - 4 * a * c}}{2a} \tag{6}$$

with L is either the upper or lower limit of the confidence interval (depending on ±) and

$$a = q^2 - z^2 * \mathrm{var}(q) \tag{7a}$$

$$b = 2 * \mathrm{cov}(p,q) * z^2 - 2 * p * q \tag{7b}$$

$$c = p^2 - z^2 * \mathrm{var}(p) \tag{7c}.$$

Variation of K_i was calculated using the following estimation (Chatfield, 1983)

$$\left[CV(K_i)\right]^2 = \frac{\mathrm{var}(K_i)}{\left[E(K_i)\right]^2} = \left[CV\left(\frac{p}{q}\right)\right]^2 \approx \left[CV(p)\right]^2 + \left[CV(q)\right]^2 = \frac{\mathrm{var}(p)}{\left[E(p)\right]^2} + \frac{\mathrm{var}(q)}{\left[E(q)\right]^2} \tag{8},$$

with CV is the coefficient of variance, and E is the estimated value.

This procedure was applied to calculate K_i-values of both dichlorvos and rainwater extracts. Total inhibitor concentrations [I] in the extracts were expressed as dimensionless concentration factors of the rainwater [ml rainwater per ml reaction mixture], whereas concentrations [I] of the dichlorvos had dimensions of [nM]. Since both K_i-values represent an inhibitor concentration with the same characteristics, the toxic potency of the rainwater sample could be expressed in terms of nmoles dichlorvos per liter rainwater by

$$C_{DEQ} = \frac{K_i(DCV)}{K_i(ex)} \tag{9},$$

with C_{DEQ} is the dichlorvos equivalent concentration of the rainwater expressed in [nmoles dichlorvos per liter rainwater], K_i(DCV) is the K_i-value of dichlorvos and K_i(ex) is the K_i-value of the extract. A 95% confidence interval for the DEQ concentration was again calculated by Fieller's theorem (equation 6).

The progressive inhibition of honeybee homogenate mediated ATC-hydrolysis and AChE mediated MIA hydrolysis was described by

$$\ln\left(\frac{V_0}{V_t}\right) = \frac{k_{+2} * [I]}{K_A + [I]} * t \tag{10}$$

(Main, 1964; Main and Iverson, 1966; Aldridge and Reinier, 1972), where V_0 and V_t are the initial reaction rates of substrate hydrolysis at t=0 and at time t after starting incubation of the enzyme with inhibitor, and [I] is the concentration of the inhibitor. K_A is the affinity constant (k_{-1}/k_{+1}) for the formation of the reversible complex, and k_{+2} is the rate of the progressing irreversible phosphorylation. Bimolecular inhibition constants ($k_i = k_{+2}/K_A$; Main, 1964) were calculated by non-linear regression based on equation 10.

Results

Progressive inhibition experiments with dichlorvos

ATC hydrolysis by honeybee homogenate was progressively inhibited with increasing incubation time and increasing concentrations of dichlorvos. In a typical inhibition curve (Figure 1A), a maximum rate of 100% at t=0 could be extrapolated for each concentration of dichlorvos. A bimolecular inhibition constant k_i was estimated of $6.3*10^5$ M^{-1} min^{-1}.

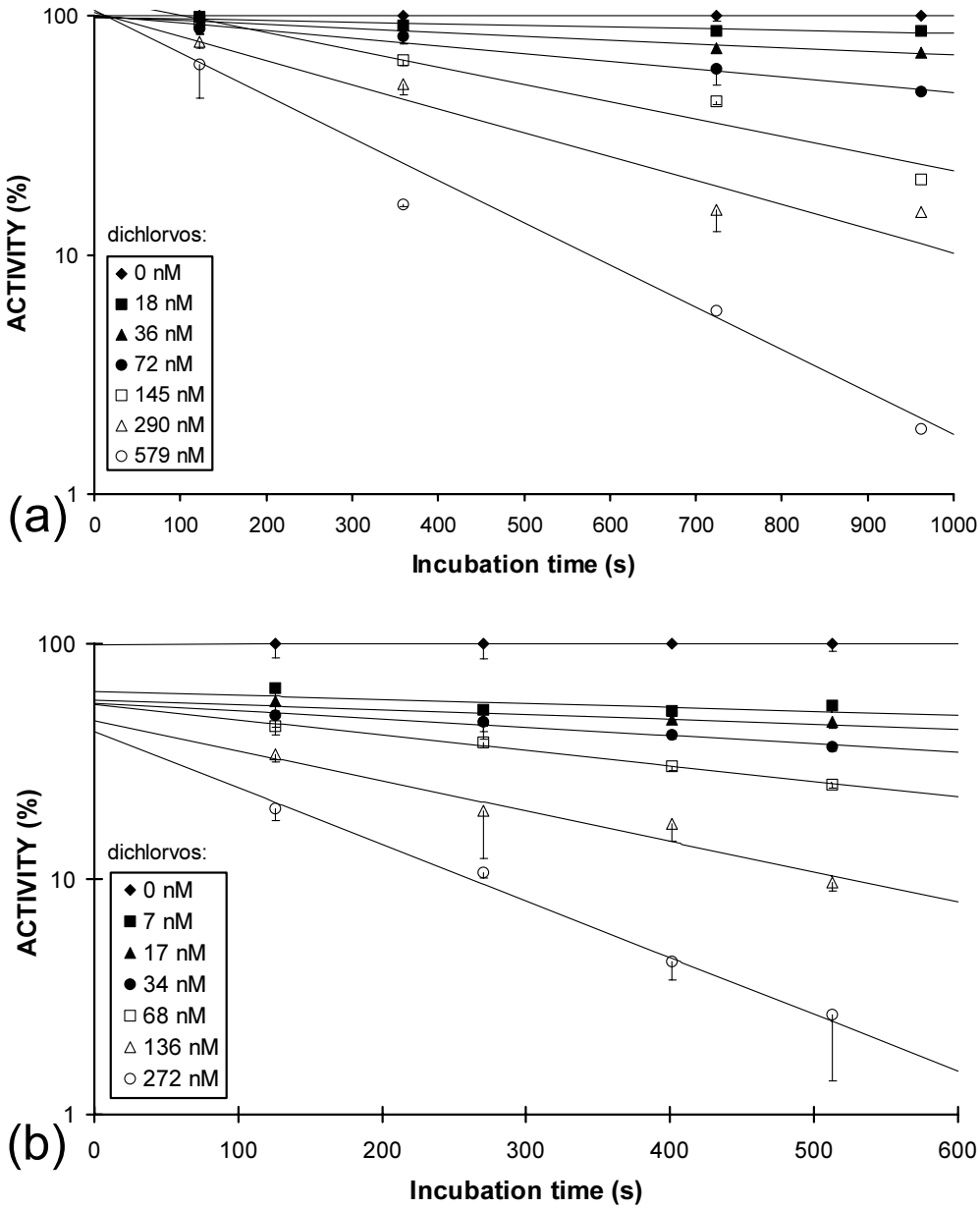

Figure 1: Progressive inhibition curves of dichlorvos for the inhibited hydrolysis of different substrates [S] by different enzymes [E]. The esterase activity is expressed as a percentage of the hydrolysis rate in the absence of an inhibitor. **(a)** [S]=ATC and [E]=honeybee homogenate (*Apis mellifera*); **(b)** [S]=MIA and [E]=honeybee homogenate.

For MIA-hydrolysis also a clear dose-inhibition relationship was found for dichlorvos (Figure 1B), but inhibition occurred at much lower dichlorvos concentrations than with ATC-hydrolysis. Furthermore, a maximum rate of 100% could be extrapolated at t=0 for none of the dichlorvos concentrations tested (Figure 1B), indicating that an enzyme-inhibitor complex is formed instantly when enzyme is added to the inhibitor. To test whether this complex formation is reversible, an experiment was performed in which concentrations of enzyme and inhibitor were similar to the experiment in Figure 1B, but incubation volumes were 5 times smaller. After incubation, the enzyme and inhibitor were diluted 5 times by a MIA-solution in P-buffer, leading to similar final concentrations of MIA (0.265 mM) and final reaction volumes (200 µl) as in the undiluted situation of Figure 1B. Although the incubation mixture was only diluted by a factor 5, the intercept (*i.e.* the distance between 100% and the intersection of the extrapolated curves on the y-vertical axis) was considerably decreased by dilution (10-20%), indicating that the formation of the enzyme-inhibitor complex is reversible (data not shown). Similar results were found with paraoxon as inhibitor.

No such intercepts were found for the progressive inhibition curves of MIA-hydrolysis by electric eel AChE incubated with dichlorvos, except for the highest dichlorvos concentration tested (1000 µg/l). Despite the fact that enzyme concentrations were used with similar MIA-hydrolyzing capacities (*i.e.* 15.2 µl of bee homogenate per ml ≡ 102 mU of eel AChE per ml), progressive inhibition of AChE mediated MIA-hydrolysis was found only at dichlorvos concentrations higher than the highest concentration tested in Figure 1B. A bimolecular inhibition constant k_i was estimated of $6.3*10^5$ M^{-1} min^{-1}.

For greatest sensitivity of the assay it was decided to further apply bee esterases and MIA as the enzyme source and its substrate, respectively. As the reversible enzyme-inhibitor complex was formed at low concentrations of inhibitor, quantification of (the kinetics of) this complex was further studied in reversible inhibition experiments with dichlorvos (Protocol II).

Reversible inhibition experiments with dichlorvos

The initial reaction rate of the honeybee esterase catalyzed hydrolysis of MIA immediately decreased with increasing concentrations of dichlorvos. As expected, no such dose-response relationship was found for ATC-hydrolysis, because no intercept was observed in Figure 1A. After reciprocal transformation of the initial reaction rate [v_0] and the concentration of MIA [S], the obtained Lineweaver-Burk plot (Figure 2) was best described

by the multiple regression model based on competitive inhibition (Equation 3; Table 1). The model is characterized by a common intercept $1/V_{max}$ on the vertical $1/v_0$-axis for all dichlorvos concentrations tested and by slopes increasing proportionally with higher concentrations of inhibitor. This model for competitive inhibition kinetics was further applied to quantify the esterase inhibiting potency of rainwater extracts, because alternative regression models based on uncompetitive or mixed inhibition kinetics were less appropriate. Besides, competitive inhibition of MIA-hydrolysis catalyzed by honeybee homogenate occurred at dichlorvos concentrations about one order of magnitude lower than progressive inhibition (*cf.* Figures 2 and 1).

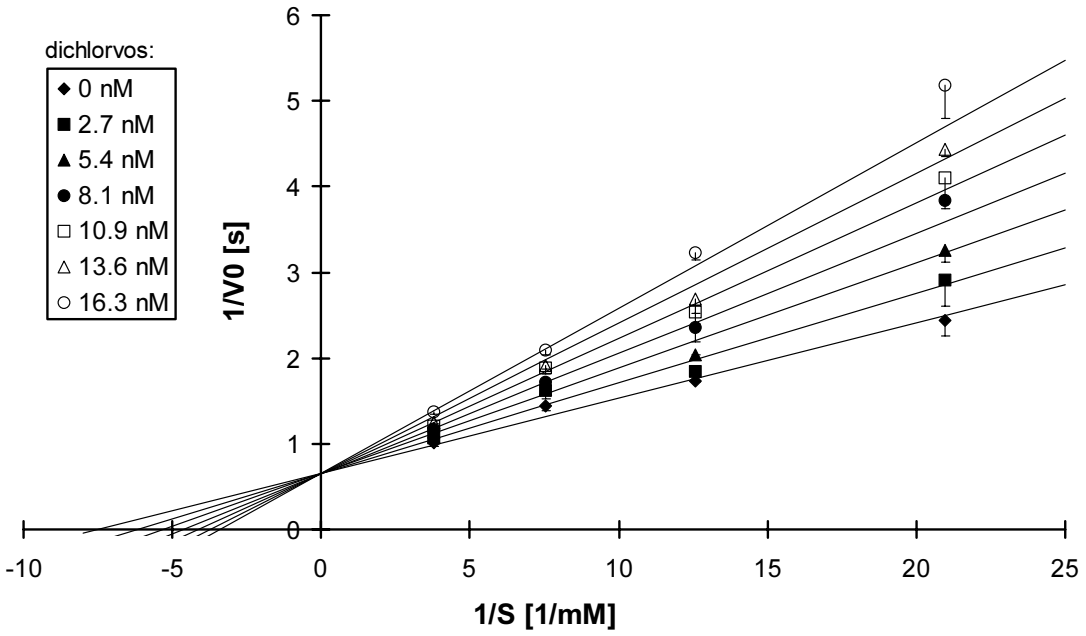

Figure 2: Double-reciprocal Lineweaver-Burk plot with multiple regression lines for the competitive inhibition by dichlorvos of MIA-hydrolysis by honeybee homogenate (*Apis mellifera*). v_0: initial reaction rate expressed as arbitrary fluorescence units (AFU) per second; S: substrate concentration (MIA)**.**

Further simplification and improvement of the sensitivity of the assay were obtained by working at room temperature rather than at 37°C and by adding 50 µl of less concentrated bee homogenate to the microtiter plate (*i.e.* 6.5 µl/ml in stead of 15.2 µl/ml). Parameter estimates for competitive inhibition of honeybee mediated MIA hydrolysis under these optimized experimental conditions are also given in Table 1.

Table 1: Test conditions, parameter estimates of the multiple regression and results of further data processing from the dichlorvos experiments.

	Figure 2	Further optimization
Concentration of bee homogenate added (µl/ml)	15.2	6.5
Concentration range of dichlorvos tested (nM)	0-16	0-13
Temperature (°C)	37	room temperature
p (mM*s)	0.0877	0.184
q (mM*s*(nM)$^{-1}$)	0.00643	0.0513
r (s)	0.654	2.69
K_m (mM MIA)	0.134	0.0684
K_i (nM dichlorvos)	13.6	3.58
V_{max} (AFU/s)	1.53	0.372
95% confidence interval K_i (nM dichlorvos)	12.1-15.5	2.97-4.25
R^2	0.974	0.953

Reversible inhibition experiments with rainwater extracts

For all four rainwater samples tested, MIA-hydrolysis by honeybee esterases was most inhibited by the first elution fraction with methanol. Consecutive elution with dichloromethane and hexane yielded fractions with much less esterase inhibiting potency, and no inhibition was detected after exposure to pure or diluted rainwater. The inhibition of MIA hydrolysis by the methanol fraction (Figure 3) and the dichloromethane and hexane fraction of a rainwater extract show clear dose-response relationships.

Based on Equation 8, dichlorvos equivalent concentrations (µg/l) were calculated for the rainwater samples (Table 2), by dividing the K_i-value of dichlorvos (0.791 µg/l ≡ 3.58 nM; Table 1) by the K_i-values of the three eluted solvent fractions of each rainwater extract. The sample of 13 June contained highest total esterase inhibiting potency. For all 4 samples, the toxic potency of the different solvent fractions decreased with each consecutive eluent (Table 2). The recoveries for the elution fraction of the spiked batch were 56% and 17% for the methanol and dichloromethane fraction, respectively, and no esterase inhibiting potency was present in the hexane fraction.

Table 2: Esterase inhibiting potency of the consecutively eluted fractions of the four rainwater samples expressed as equivalent concentrations of dichlorvos (ng/l) in rainwater (95% confidence intervals are given in parentheses).

Fraction	April 29, 1997	May 7, 1997	May 15, 1997	June 13, 1997
Methanol	12.6 (10.0-16.2)	64.6 (53.2-77.3)	25.1 (19.5-32.9)	125 (96.6-165)
Dichloromethane	3.72 (2.93-4.73)	21.9 (17.8-26.7)	7.17 (5.47-9.69)	3.58 (2.78-4.68)
Hexane	-[a]	1.27 (0.96-1.72)[b]	3.86 (2.99-5.06)	1.96 (1.50-2.63)

[a]: not determined

[b]: the concentration of 1.27 (0.96-172) ng/l was considered to be an indicative value, as the underlying K_i-value was higher than the concentration range tested.

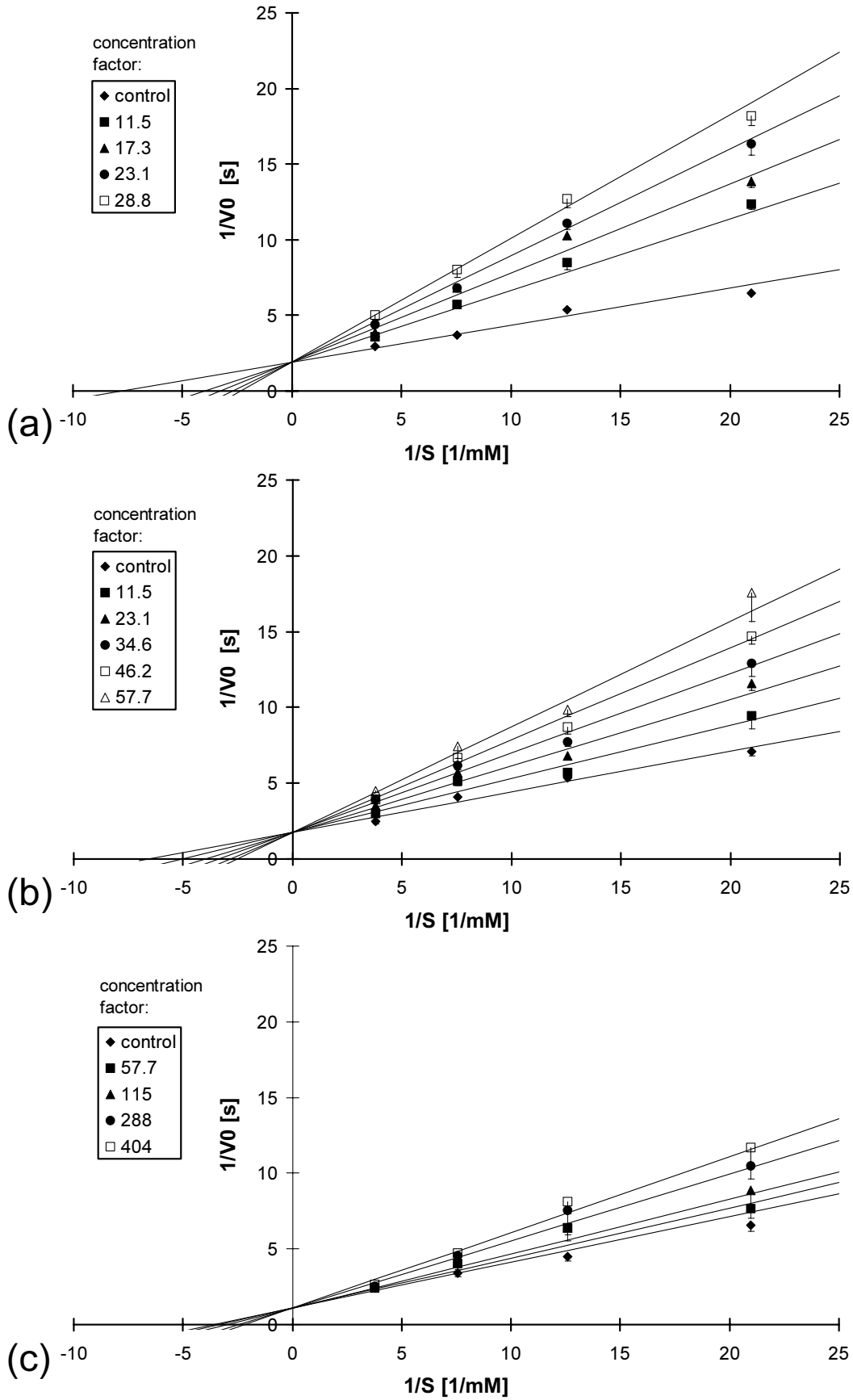

Figure 3: Double-reciprocal Lineweaver-Burk plots with multiple regression lines for inhibition of honeybee homogenate mediated MIA hydrolysis by extracts of the rainwater sample of May 7, 1997. **(a)** methanol eluate; **(b)** dichloromethane eluate; **(c)** hexane eluate.

Discussion

Selection of the most sensitive test conditions

Remarkably, honeybee esterase mediated MIA-hydrolysis was instantly inhibited at very low concentrations of dichlorvos; 37% and 53% of inhibition was extrapolated at t=0 for concentrations of 7 nM and 136 nM, respectively (Figure 1B). This instant inhibition showed a clear dose-response relationship as intercepts increased with increasing concentrations of dichlorvos. Moreover, the decrease of the intercepts after dilution indicates that inhibition is due to the formation of a reversible Michaelis-complex (Aldridge and Reinier, 1972), which is formed instantly when incubation is started, and can be quantified by Michaelis-Menten kinetics.

Irrespective of the enzyme source and substrate used, progressive inhibition by dichlorvos was very slow at these concentrations. Using higher concentrations of dichlorvos, bimolecular inhibition constants of $6.3*10^5$ and $1.1*10^5$ M^{-1} min^{-1} were determined for honeybee esterase mediated ATC-hydrolysis and AChE mediated MIA-hydrolysis, respectively. These k_i-values are within the same range as reported by De Bruijn and Hermens (1993) and Xu and Bull (1994), who used ATC as a substrate for purified AChE and determined k_i-values in the range of 10^4-10^6 M^{-1} min^{-1} for 21 of all 23 (oxon-analogues of) organophosphates studied. In practice this means that for more than 90% of the inhibitors tested, concentrations ranging from 70-7000 nM are required to reach 50% of inhibition within 10 min of incubation ($IC50_{10min}$). To quantify the reversible inhibition complex, a maximum dichlorvos concentration of 16 nM was tested (Figure 2), which is 4.4-440 times less than this range of $IC50_{10min}$-values. Considering the goal of the present study to select optimum test conditions for measuring low concentrations of esterase inhibitors in small samples, it was decided to quantify the toxic potency of rainwater by the reversibly inhibited hydrolysis of the substrate MIA by esterases present in honeybee homogenate.

Substrate-specificity of esterases

The homogenate of honeybee heads contains not only AChE, but also many unspecific cytosolic carboxylesterases. Apparently, using both specific ATC and non-specific MIA as a substrate allows discrimination between the activity of (acetyl-)cholinesterase and carboxylesterases in the honeybee homogenate, because different combinations of substrates and enzymes have different inhibition kinetics. As ATC-hydrolysis by honeybee homogenate

showed similar kinetics of progressive inhibition by dichlorvos as ATC-hydrolysis by purified AChE (*c.f.* Figure 1A and for instance results of De Bruijn and Hermens [1993]), it is hypothesized that ATC is actually hydrolyzed by the (acetyl-)cholinesterase present in honeybee homogenate, and not by the non-specific carboxyl-esterases. This is confirmed by results from Bitondi and Mestriner (1983), who found no cholinesterase activity for 6 different isozymes of carboxylesterases isolated from homogenate of *Apis mellifera* using ATC as substrate.

For organophosphate compounds, reversible inhibition of carboxylesterases from honeybee homogenates with non-specific substrates has been described previously. Frohlich *et al.* (1990) found mixed mechanisms of competitive and uncompetitive inhibition for the alfalfa leaf-cutting bee *Megachile rotundata* and Spoonamore *et al.* (1993) found that inhibition mechanisms were highly competitive in nature (explicitly for dichlorvos) for the honeybee *Apis mellifera*. In both studies, *p*-nitrophenylacetate was used as a non-specific substrate. In the study of Bitondi and Mestriner (1983), this same substrate was exclusively hydrolyzed by only one carboxylesterase isozyme (number 3) of the 6 isolated isozymes of carboxylesterases (Figure 4). This isozyme 3 also showed highest hydrolysis activity for the substrates indoxyl acetate and bromo-indoxyl acetate, which are closely related to MIA.

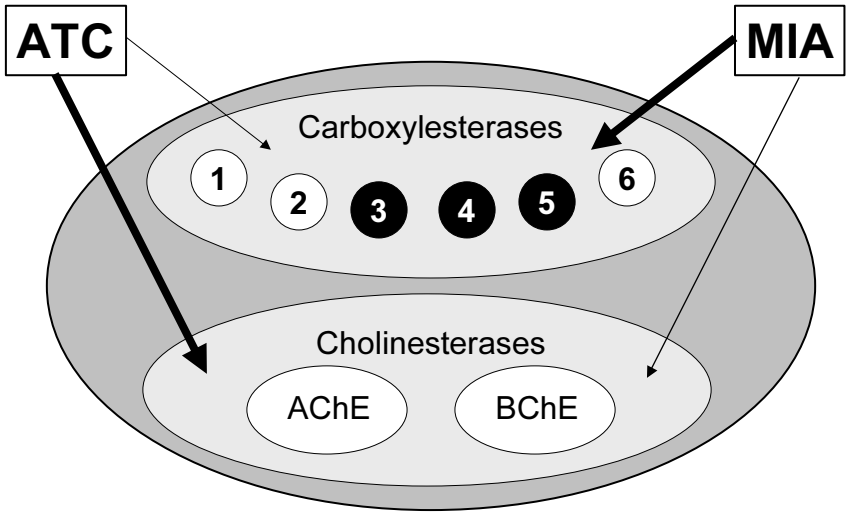

Figure 4: Schematic classification of all esterases present in the homogenate of heads of honeybees (*Apis mellifera*). The width of the arrows qualitatively indicates the affinity of the esterases for the substrate (ATC or MIA) involved. At least 6 different isozymes (1-6) have been isolated by Bitondi and Mestriner (1983) from the pool of cytosolic carboxylesterases of which none exposed any cholinesterase activity and numbers 3-5 are able to hydrolyze indoxyl acetate and bromo-indoxyl acetate, which are closely related to MIA. Isozyme 3 is also capable of hydrolyzing *p*-nitrophenol, a substrate of which the hydrolysis by honeybee homogenate is also competitively inhibited by organophosphates (Spoonamore *et al.*, 1993).

Based on the similarity in reversible competitive inhibition kinetics between Spoonamore *et al.* (1993) and our study, it can be hypothesized that MIA-hydrolysis is mainly catalyzed by the unspecific carboxylesterases present in the homogenate rather than by (acetyl-)cholinesterases (Figure 4). Apparently, these carboxylesterases are also progressively inhibited at high concentrations of inhibitor, but have different inhibition kinetics than AChE at low inhibitor concentrations, due to a higher affinity constant K_A. The experiments with purified AChE indicate that AChE in the homogenate has much lower affinity for MIA than for ATC (Figure 4). AChE-catalyzed MIA-hydrolysis is still progressing 1500 seconds after substrate addition (data not shown), whereas in case of equal affinity the 53 nmol of MIA present in each microtiter well is hydrolyzed within 624 seconds by 5.1 mU AChE-activity.

Application of the esterase inhibition assay to rainwater samples

Already in the first elution step of the extraction disk with methanol, most esterase-inhibiting potency of the rainwater was collected (Table 2). The dichloromethane fraction contained much less potency and the hexane fraction contained negligibly amounts. Therefore, elution with methanol and dichloromethane is sufficient to collect all toxic potency from the extraction disk.

The lowest estimated concentration of 2.0 ng dichlorvos-equivalents per liter rainwater (hexane elution fraction of June 13, 1997, Table 2) was calculated using equation 9, with K_i-values of 0.79 mg/l and 404 ml/ml for dichlorvos and rainwater, respectively. Based on the latter value, 2 ng/l in the rainwater is equivalent to 0.8 µg/l in the microtiter plate. Assuming this to be the highest concentration required in the concentration series and taking into account that final 1-propanol concentrations should be smaller than 0.09%, the sensitivity of the assay can be increased by reducing the dilution volume of the extract from 2 to 0.4 ml and by reducing the volume of 1-propanol as a carrier from 10 to 5 µl. Thus, the limit of detection is decreased to 1.4 ng/l dichlorvos-equivalents in 6 l samples and to 14 ng/l in 0.6 l samples.

The maximum dichlorvos-equivalent concentration of 125 ng per liter of rainwater determined in this study (Table 2) exceeded 179 times the Maximum Permissible Concentration (MPC) of dichlorvos in surface water (0.7 ng/l; Crommentuijn *et al.*, 1997). However, this concentration is still 3.2-36 times lower than the maximum concentrations of the individual compound dichlorvos previously reported in rainwater in The Netherlands collected in different locations and in different seasons (Fleverwaard, 1993; Province of South-Holland, 1994; Baas and Duyser, 1997). Further validation of the assay with chemical

analyses of the rainwater samples has been performed, and good correlations have been found both in time and in place for chemically analyzed organophosphate compounds and esterase inhibiting potencies in rainwater extracts (Chapter 4).

Expressing the esterase inhibiting potency of the extracts in dichlorvos equivalent concentrations assumes that the complex mixture of inhibitors in the extract behaves as a virtual single compound with its own specific K_i-value. The proportional increase (R^2>0.98) of the slopes of the Lineweaver-Burk plots with increasing concentrations of inhibitor-mixture (*i.e.* rainwater extract or the mixture of 20 organophosphate insecticides) supports this assumption.

Conclusions

N-methylindoxylacetate (MIA) and carboxylesterases from honeybee homogenate is the most sensitive combination of substrate and enzyme, respectively, to determine the esterase inhibiting potency of (a mixture of) organophosphate and carbamate insecticides. In combination with solid-phase extraction, a bio-assay based on the inhibited hydrolysis of MIA by carboxylesterases allows the rapid quantification of the esterase inhibiting potency in rainwater as low as 2 ng dichlorvos-equivalents per liter. The sensitivity of carboxylesterases from honeybee homogenate is attributed to the instant formation of a reversible Michaelis-Menten complex between the inhibitor and the esterases, which is not formed between the inhibitor and purified AChE. Based on the inhibitor constants (K_i) of the model inhibitor dichlorvos and the rainwater extracts, the esterase inhibiting potency of the rainwater can be recalculated into an equivalent concentration of dichlorvos. Most inhibitory capacity is present in the hydrophilic fraction of rainwater extracts. In the 4 rainwater samples tested, esterase-inhibiting potency was equivalent to dichlorvos concentrations of 12-125 ng/l.

Acknowledgements

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Chapter 4

Biological and chemical analysis of the toxic potency of pesticides in rainwater

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Timo Hamers
Mathijs GD Smit
Albertinka J Murk
Jan H Koeman

Esterase enzymes present in a crude homogenate of crushed heads of honeybees (*Apis mellifera*) proved to be very sensitive to inhibition by organophosphate and carbamate insecticides (Chapter 3). The specimen on this picture carries a grain of pollen at its back legs.

Abstract

A newly developed method for measuring the integrated esterase inhibiting potency of rainwater samples was applied in practice, and the results are compared to the toxic potency calculated from concentrations of 31 organophosphate and carbamate pesticides, out of a total of 66 chemically analyzed pesticides. In addition, the general toxic potency of the rainwater samples was evaluated in a microtiter bioluminescence assay with *Vibrio fischeri* bacteria. Rainwater samples were collected over four consecutive 14-day periods in both open and wet-only samplers. The esterase inhibiting potency of the open rainwater samples (expressed as ng dichlorvos-equivalents/l) corresponded well with the chemical analyses of the rainwater samples collected by both types of samplers (r=0.83-0.86). By far the highest esterase inhibiting potency was found in a sample collected in an area with intense horticultural activities in June, and was attributed to high concentrations of dichlorvos, mevinphos, pirimiphos-methyl and methiocarb. The esterase inhibiting potency of this sample was equivalent to a dichlorvos concentration of 1380 ng/l in the rainwater, which is almost 2000 times higher than the maximum permissible concentration of dichlorvos set for surface water in The Netherlands. Maximum individual concentrations of dichlorvos and pirimiphos-methyl even exceeded the EC_{50} for Daphnia, suggesting that pesticides in rainwater pose a risk for aquatic organisms. Not all responses of the bioluminescence-assay for general toxicity could be explained by the analyzed pesticide concentrations. The bio-assays enable a direct assessment the toxic potency of all individual compounds present in the complex mixture of rainwater pollutants, even if they are unknown or present at concentrations below the detection limit. Therefore, they are valuable tools for prescreening and hazard characterization purposes.

Introduction

Airborne pesticides originating from local use or (long-range) atmospheric transport from diverse sources form complex mixtures in the atmosphere. Evaporation during application or from soil and plant surfaces after application is the main route for pesticides to enter the atmosphere, but spray drift and wind erosion are also important processes. Over the past few years, several research groups have demonstrated high concentrations of pesticides in rainwater in The Netherlands, which may significantly contribute to pollution of surface water. In The Province of Flevoland, pesticide concentrations in rainwater were on average

two times higher than in simultaneously collected samples of surface water (Fleverwaard, 1993). For four locations with different agricultural activities in the Province of South-Holland, average rainwater concentrations (ng/l) have been reported to be 5-95 for dichlorvos, 29-56 for lindane, 6-18 for parathion-ethyl, 0-12 for mevinphos, 2-12 for pyrazophos, 5-9 for β-endosulfan, 1-5 for malathion and 3-4 for hexachlorobenzene (Province of South-Holland, 1994). These values exceeded the maximum permissible concentration (MPC) of these pesticides set for surface water in the Netherlands in 1994 by a factor 2 (mevinphos) to 50 (dichlorvos).

For a hazard evaluation, the concentrations of the individual compounds are currently compared to their critical concentration set for surface water. For the purpose of regulatory environmental risk assessment, an international workshop recently proposed an additional approach based on comparing the predicted or chemically analyzed concentrations of individual pesticides in the atmosphere or deposition to their critical concentration based on their chemical characteristics and toxicological endpoints (Guicherit *et al.*, 1999). However, this procedure may lead to an underestimation of the integrated toxic potency, as not all pesticides are chemically analyzed, critical concentrations are not known for all pesticides, and combination effects may exist. This problem may be solved by measuring the integrated toxic potency of rainwater samples in small-volume bio-assays, indicating either specific or general toxicity.

The goal of the current paper was to compare the integrated toxic potency of rainwater extracts to the chemically analyzed concentrations of individual pesticides present in the rainwater. Rainwater was collected in four sampling periods of 14 days in the summer of 1997 at two locations in the Province of South-Holland. Both open samplers and wet-only samplers were used. The toxic potency of the samples from the open samplers was determined in two small-scale bio-assays.

1. The esterase inhibition assay responds specifically to organophosphate (OP) and carbamate (CARB) insecticides, which made up almost 70% of the total amount of insecticides sold in The Netherlands in 1998 (RIVM/CBS, 1999). These compounds are an important group of rainwater pollutants, as they are toxic to a broad spectrum of species and are efficiently scavenged from the atmosphere by wet deposition, due to their relative polar character. The assay was specifically optimized for analyzing rainwater samples as has been described elsewhere (Chapter 3).

2. The small scale bioluminescence assay is a non-specific toxicity assay, which measures general inhibition of respiration in *Vibrio fischeri* marine bacteria. It is comparable to the Microtox-assay first described by Bulich (1979), but was adapted to fast and small volume testing.

All samples were also chemically analyzed for carbamate pesticides, organophosphate insecticides, N-, P- or Cl-containing insecticides and organochlorine pesticides. Both the toxic potency of the rainwater determined in the esterase inhibition assay and the analyzed pesticide concentrations are calculated into dichlorvos-equivalent concentrations, whereafter the measured toxic potency is compared to the calculated toxic potency based on the individual pesticide concentrations.

Materials and Methods

Collection of rainwater samples

Rainwater was sampled at two locations in the Province of South-Holland, The Netherlands:

1. Location HORT is characterized by intensive greenhouse horticultural activities in the so-called Westland area. The samplers were situated at the Research Station for Floriculture and Glasshouse Vegetables in Naaldwijk.
2. Location BACK is regarded to be a relative background area for the Dutch situation, especially when compared to HORT. The samplers were situated at the shore of Lake Nieuwkoopse Plassen in Noorden.

On both sampling locations, two methods of sample collection were performed, *i.e.* by open collectors, which are open all the time, and by a wet-only collector, which is open only during rainfall. Samples from both open and wet-only collectors were chemically analyzed, so that the contribution of dry deposition to the total deposition of pesticides could be estimated. Only samples from open collectors were analyzed in the bio-assays, because samples from wet-only collectors were too small to allow both chemical analyses and bio-assays.

Open sampling was performed in 10 open samplers consisting of a clean amber bottle (2.5 l), at 1.5 m above ground level. On top of each bottle a smooth polyester funnel (∅=24 cm) was placed. Wet-only sampling was performed with a REG-100 sampler (Van Essen, Delft), which consists of a glass bottle (5 l) connected to a glass funnel (400 cm^2). Both bottle and funnel are placed in a dark container with a lid, which opens when a rain sensor

connected to the lid is moistened. Samples were collected from the field sites after 14-day sampling periods at 28 May, 11 June, 25 June and 9 July 1997. On arrival at the laboratory, pH and volume of each sample was measured and samples were stored in the dark at 4°C. On the next day, each sample was divided into portions needed for the different analyses, according to priorities set beforehand (Table 1). Open samples were both chemically and biologically analyzed, whereas wet-only samples were only chemically analyzed.

Table 1: Reserved sample volumes and priorities set for further processing and analyses of the subsamples.

Analysis	Volume required (ml)	Priority[a]	
		Open sample	Wet-only sample
bioluminescence assay on sample dilutions	10	1	
chemical analysis N-methyl carbamate pesticides	4	2	1
chemical analysis organophosphate pesticides	1000	3	2
chemical analysis N, P, Cl containing pesticides	250	4	3
chemical analysis organochlorine pesticides	250	5	4
bioluminescence assay on sample extracts	440	6	
esterase inhibition assay on sample extracts	5200	7	

[a]: 1 means first priority, 2 - second priority, etc

Chemical analyses

Analyses of N-methyl carbamate pesticides (CARB) and N-, P- or Cl-containing compounds were performed by the Central Laboratory of the Water Authority Hollandse Eilanden en Waarden (ZHEW, Dordrecht). The analysis of carbamate compounds (CARB) is based on NEN-6403 (1997). Subsamples of 4 ml of rainwater were acidified by 100% glacial acetic acid (p.a.) and led over a SPE-cartridge with polystyrenedivinylbenzene as active phase (PLRP-S [15-25 µm; 10mm×2 mm]; Spark-Holland). Compounds were eluted from the cartridge and separated on a second analytical column with C18 as active phase (Alltech Carbamate 5µm; 250mm×4.6 mm, Alltech). The separated N-methylcarbamates were then hydrolyzed to methylamines on a third column (Aminex A27). After addition of a reagent containing o-phthalaldehyde and 2-mercapto-ethanol, the methylamines transformed into an iso-indol, which was detected fluorimetrically (λ_{ex}=340 nm; λ_{em}=445 nm). The analysis of N-, P-, or Cl-containing compounds was based on a protocol that was especially developed by ZHEW. To a subsample of 250 ml of rainwater, 25 g of NaCl (p.a., Merck) was added, whereafter liquid-liquid extraction was performed by 3 times intense shaking with ethylacetate (Baker Ultra Resi-Analyzed). The extract was dried with Na_2SO_4 (p.a., Merck)

and evaporated in a Turbovap to a final volume of 0.5 ml. The compounds were detected by GC-MS (DB-5MS column 0.25 µm; 30m×0.25mm, J&W).

Organophosphate (OP) and organochlorine (OC) compounds were analyzed by the Laboratory of the Polderboard Delfland (HHR-D, Delft). The analysis of OP insecticides was based on a method described in VPR-C88-18 (1988). Prefiltered (seasand; Merck) subsamples of 1000 ml of rainwater were extracted on a Solid Phase Extraction column (2000 mg C18; JT Baker), with consequent elution with 5×2 ml of acetone (nanograde; Promchem). The extract was dried with Na_2SO_4 (p.a., Merck), and evaporated to a final volume of 1 ml in a Kuderna-Danish bottle (70°C). The OP insecticides were detected by GC (30 m fused silica column with DB-1701 as stationary phase; J&W, Bester b.v.) with a flameless N,P detector.

The analysis of OC insecticides was based on a method described in NEN-5734 (1995) and NEN-6406 (1990). Subsamples of 250 ml of rainwater were extracted by means of liquid-liquid extraction with hexane (nanograde; Prochem). The extract was dried with Na_2SO_4 (p.a., Merck), and evaporated to a final volume of 0.5-1.0 ml in a Kuderna-Danish bottle (90°C). After injection, samples were directly divided in two parts, which were led over columns with different polarities (*i.e.* 30 m fused silica column with DB-5 or DB-1701 as stationary phase; J&W, Bester b.v.). After elution, detection was performed by Electron Capture Detection.

The detection limits (DL) and limit of quantification (LoQ) was defined as three times and ten times, respectively, the standard deviation of the concentration of a spiked sample that was analyzed in ten-fold. For all compounds, LoQs have been listed in Tables 4-7.

Fourteen pesticides (OP and OC) were analyzed both by HHR-D and by ZHEW. Analytical results were in good agreement in case both laboratories detected a pesticide. In many cases, however, a pesticide was detected by only one laboratory, and not by the other, probably due to differences in rainwater volumes reserved for the analyses and in methods of extraction and detection. With respect to these 14 pesticides, further Figures and calculations are based on the analyses by HHR-D, as they had a lower detection limit for esterase inhibiting OP-insecticides.

Sample preparation for bio-assays

No pre-treatment was given to the sub-samples that were tested directly and diluted in the *Vibrio fischeri* bioluminescence assay. Prefiltered (seasand; Merck) subsamples of 5600 ml rainwater were extracted on a Solid Phase Extraction column (2000 mg C18: JT Baker) (Figure 1), which was eluted consecutively with nanograde acetone, dichloromethane and

hexane (Prochem) to yield fractions containing compounds with different polarities. Each solvent fraction was split into two parts, corresponding with 2600 ml and 220 ml of rainwater to be tested in the esterase inhibition assay and the *V. fischeri* bioluminescence assay, respectively. Additionally, the equivalent of 2820 ml of rainwater of the three solvent fractions were mixed together for the so-called "combination fraction", which was also divided into two parts corresponding with 2600 ml and 220 ml of rainwater. All extract fractions were evaporated under a gentle N_2-flow at 30°C and dissolved in 10 µl of 1-propanol (99+% Acros).

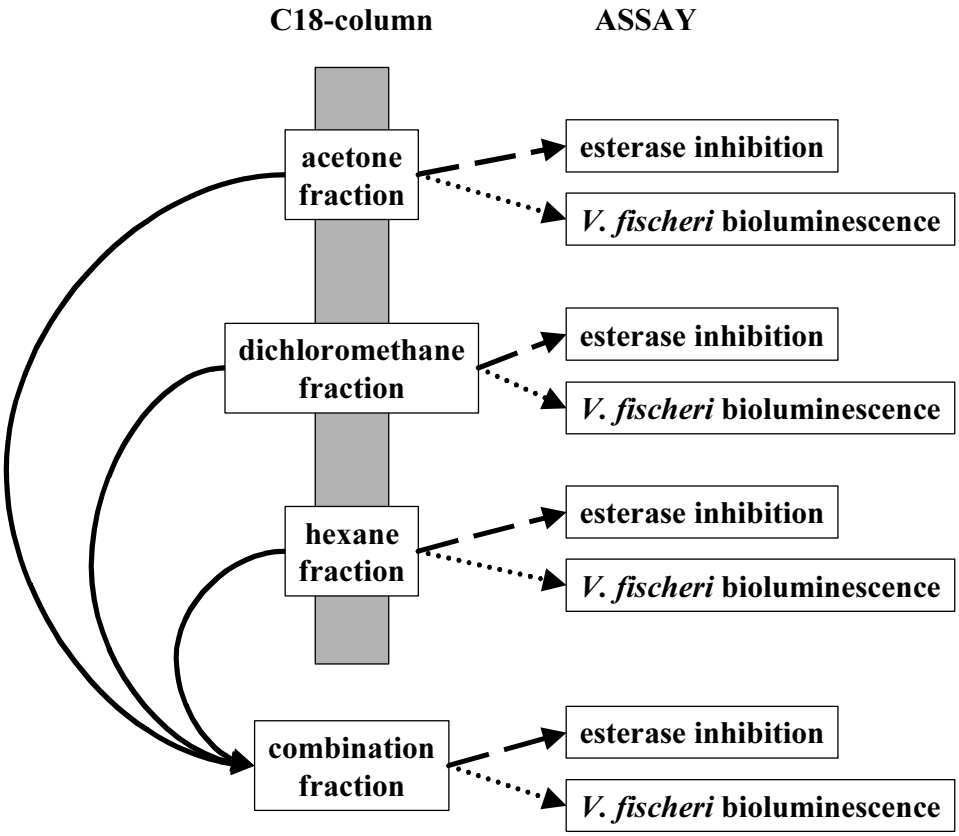

Figure 1: Scheme for splitting the rainwater subsamples intended for bio-assay testing into different fractions: 5640 ml of rainwater were extracted on a C18-column, which was eluted with acetone, dichloromethane and hexane, consecutively. Solid, dashed, and dotted arrows refer to extracts equivalent to 2820, 2600, and 220 ml rainwater, respectively.

Chemicals

N-Methylindoxylacetate (MIA) (1-H-indol-3-ol, 1 methyl-acetate [ester]) was obtained from Sigma and was kept in a stock solution of 1 mg/ml (5.3 mM) in methanol (HPLC Labscan) at -20°C in the dark. Dichlorvos (2,2-dichlorovinyldimethyl-phosphat) was used as a standard organophosphate insecticide and was obtained from Riedel-De Haën. Stock solutions were made in methanol (HPLC Labscan) and kept at 4°C in the dark. Phosphate

buffer (0.1M; pH=7) was made by mixing 0.1 M solutions of KH_2PO_4 and K_2HPO_4 in demineralized water.

Esterase inhibition assay

The esterase inhibition assay is based on blocking of esterases by organophosphate and carbamate insecticides, thus preventing the hydrolysis of substrates. The specially adapted assay (Chapter 3) is based on the inhibition of esterases from heads of working bees (*Apis mellifera*) by insecticides extracted from rainwater. The inhibited hydrolysis of the substrate N-methylindoxylacetate (MIA) can be described by Michaelis-Menten kinetics for reversible inhibition.

Honeybees were obtained from Wageningen University – Laboratory of Entomology. Bees were anaesthetized with CO_2, frozen to death at -20°C and decapitated. Eighteen heads were homogenized in 6 cycles, consisting of adding 2 ml P-buffer to the 18 heads, homogenizing the heads with a laboratory homogenizer (Potter tube) and collecting the crude homogenate. After the last cycle the crude homogenate (about 12 ml) was centrifuged (5 min; 4388 g). The supernatant was frozen at -20°C in portions of 250 and 500 µl. Several portions of bee homogenate were used for the experiments described in this paper. Protein levels were measured by the method of Bradford (1976) and ranged from 1.3 to 1.5 g/l.

On each experimental day, dilution series of rainwater extracts and pesticide standards were freshly made in P-buffer. Extracts from 2600 ml of rainwater in 10 µl of 1-propanol were diluted in 2 ml of P-buffer. From this solution, a linear series was made of five dilutions (final volume = 100 µl) in a 0.5% 1-propanol solution in P-buffer, which was further diluted by adding 550 µl of P-buffer. Consequently, typical concentration factors of rainwater in the series ranged from 40 to 200 in 0.08% 1-propanol. Depending on the esterase inhibiting potency of the samples, higher or lower concentrations were required. Shortly before the experiment, the bee homogenate was thawed on ice and diluted 6.5 µl/ml in P-buffer. Simultaneously, the substrate MIA was diluted from the 5.3 mM stock-solution in methanol to 0.95, 1.6, 2.7 and 5.3 mM in methanol.

The experiments were performed in 96-well plates. The rate of MIA-hydrolysis was determined in a CytoFluor™ 2350 platereader (Millipore) by measuring the increase in fluorescence caused by the reaction product N-methyl indoxyl (70 s time intervals; λ_{ex}=418 nm; λ_{em}=500 nm). 90 µl P-buffer and 50 µl of diluted rainwater extracts were added to the plate. At t=0, 50 µl of diluted bee homogenate was added. Immediately, 10 µl of different

concentrations of MIA were added and fluorescence measurements were started. The maximum hydrolysis rate and the spontaneous, non-enzymatic MIA-hydrolysis rate were determined in the absence of an inhibitor and honeybee homogenate, respectively. In the end, the 96-well plate contained typical concentrations of 10-50 times concentrated rainwater.

Data analysis was performed as fully described in Chapter 3, using Michaelis-Menten kinetics for competitive inhibition. The esterase inhibiting potency of the rainwater samples was first quantified as the inhibitor constant K_i, which was calculated using a multiple linear regression model according to the Lineweaver-Burk equation. Next, the esterase inhibiting potency was expressed into dichlorvos equivalent concentrations, by dividing the K_i-value of dichlorvos by the K_i-value of the rainwater sample.

Assuming that the joint toxicity of esterase inhibiting insecticides can be described by concentration addition, the chemically analyzed OP and CARB concentrations of each sample were calculated into a single dichlorvos-equivalent concentration C_{DEQ}, based on the ratio of the topical LD_{50}-values for honeybee *A. mellifera* for each compound relative to dichlorvos. The equation

$$C_{DEQ} = \sum_i \left(C_i * \frac{LD_{50\,DCV}}{LD_{50\,i}} \right)$$

was used for this purpose, where DCV is dichlorvos and i is an individual OP or CARB pesticide, of which the concentration C_i is transformed into its dichlorvos-equivalent concentration. Topical LD_{50}-values of honeybees for the OP and CARB insecticides (Table 2) were kindly provided by Dr. P. Oomen (Plant Protection Service, Wageningen NL) from a personal database. Analyzed OP or CARB pesticides, for which no topical LD_{50}-values were available, had to be omitted from the DEQ calculation.

Bioluminescence-assay

Extracts from 220 ml of rainwater in 10 µl of 1-propanol were diluted to 2 ml in demineralized water. Next, 200 µl of a 22% (w/w) NaCl-solution was added, resulting in 100 times concentrated rainwater in a solution with 2% NaCl and 0.45% 1-propanol. This concentration was further diluted in a 2% NaCl and 0.45% 1-propanol solution into an exponential dilution series with steps of √10. Of each concentration, 100 µl was added in duplicate to the 96-well plate. A $CuSO_4$-concentration series (1.5-10 mg Cu^{2+}/l) in 2% NaCl was used as a positive control.

Vibrio fischeri bacteria were obtained as freeze-dried Microtox® Acute Reagent (Microbics) from Petromation, NL. At 4°C, batches of reagent were split into six subbatches of 27 mg in sterile amber vials. Subbatches were flushed with N_2 and kept at –20°C. As the bacteria turned out to be sensitive to light, the laboratory was darkened before the bacteria were reconstituted. On each test day, 166 µl of nanopure grade water (4°C) was added to each subbatch, and the suspension was further diluted into 8.33 ml of a 2% NaCl-solution.

Bioluminescence of the *V. fischeri* bacteria was measured in a Labsystems Luminoscan RS luminometer with automatic injector. The diluted bacteria suspension was connected to the dispenser and placed in the dark in the luminometer. First, the condition of the bacteria was tested. Within 300 s, 100 µl of a vital bacteria suspension added to 100 µl of a 2% NaCl control solution should display a very fast increase in bioluminescence at the beginning, reach a maximum and decline very slowly. Then, 100 µl of the carefully mixed bacteria suspension were resuspended and 100 µl were injected to each well with a test solution. Bioluminescence was measured exactly 6.5 min after injection of the bacteria. The bacteria in the 96-well plate were exposed to 50, 16, 5, 1.6 and 0.5 times concentrated rainwater.

Table 2: Topical LD_{50}-values of OP and CARB insecticides used for recalculation of chemical analyses into dichlorvos-equivalents[a].

Compound	Topical LD_{50} (µg per bee)
azinphos-methyl	0.06
bromophos-ethyl	0.44
bromophos(-methyl)	3.96
carbaryl	1.3
carbofuran	0.16
chlorfenvinphos	4.1
chlorpropham	> 16
demeton-S-methyl	0.2
diazinon	0.22
dichlorvos	0.07
dimethoate	0.12
fenthion	0.15
fosfamidon	0.32
malathion	0.27
methomyl	1.51
mevinphos	0.07
oxamyl	0.31
parathion-ethyl	0.11
parathion-methyl	0.17
pirimicarb	> 54
pirimiphos-methyl	0.39
propoxur	0.72
pyrazophos	0.25
tetrachlorvinphos	1.37
tolclophos-methyl	> 100
triallate	> 16
triazophos	0.06

[a]: *source*: P Oomen, Plant Protection Service, Wageningen NL

Results

Rainfall

For both sampling locations, wet deposition data (mm rainfall per 14-day sampling period) are shown in Table 3. Except for the second sampling period (06/11), sufficient rainwater was collected during all other periods to perform all chemical analyses and bio-assays mentioned in Table 1. In the second sampling period, insufficient rainwater was collected in the wet-only samplers to analyze organophosphate insecticides in BACK and organochlorine pesticides in HORT and BACK.

Table 3: Rainfall (mm) on both sampling locations during the four 14-days lasting sampling periods.

Sampling period	Sample code	HORT	BACK
14 May - 28 May 1997	05/28	33.4	51.7
28 May-11 June 1997	06/11	22.2	9.5
11 June -25 June 1997	06/25	28.4	32.2
25 June – 9 July 1997	07/09	74.1	51.3

Chemical analyses

From the 66 pesticides and pesticide metabolites analyzed, 48 were detected in the rainwater samples, of which 28 were above the limit of quantification (Tables 4-7). Concentrations between the detection limit and the limit of quantification are mainly indicative, and do not allow for a reliable quantification.

Many pesticides followed a specific pattern in time, either characterized by a peak concentration in one of the sampling periods or by a constant concentration during all sampling periods (Figure 2). Gamma-HCH (lindane, OC insecticide; Figure 2A) and chlorpropham (CARB herbicide) were mainly found in the first sampling period (05/28). For gamma-HCH, similar concentrations were found on both locations, whereas for chlorpropham higher concentrations were found in HORT.

In the second sampling period (06/11), peak concentrations were found for atrazine (triazine herbicide; Figure 2B), carbaryl (CARB insecticide), and disulfoton and parathion-ethyl (both OP insecticides and acaricides). Remarkably, all 4 pesticides were found at higher concentrations in samples from BACK than from HORT. Furthermore, peak concentrations of the OP compounds were only found in the samples collected with open samplers, whereas they were not detected in the wet only samples.

Table 4: Results of the chemical analysis of carbamate pesticides in the rainwater samples (µg/l) performed by ZHEW[a].

Compound	LoQ	Open sampler								Wet-only sampler							
		HORT				BACK				HORT				BACK			
		05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09
aldicarb	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
aldicarbsulfon	0.05	-	-	*0.01*	*0.01*	-	-	-	-	-	-	-	-	-	-	-	-
aldicarbsulfoxide	0.10	-	-	-	-	-	-	-	-	-	-	-	-	**0.38**	-	-	-
carbaryl	0.05	-	*0.02*	*0.02*	*0.01*	-	*0.045*	*0.01*	-	-	*0.01*	-	-	-	*0.03*	*0.01*	-
carbofuran	0.05	-	-	-	*0.01*	-	-	*0.01*	-	-	-	-	*0.01*	-	-	-	-
3-hydroxycarbofuran	0.05	-	-	**0.62**	**0.26**	-	-	*0.01*	-	-	-	**0.58**	*0.09*	-	-	-	-
methiocarb	0.10	-	*0.03*	**0.59**	*0.05*	-	-	*0.01*	-	-	*0.01*	**0.39**	*0.04*	-	-	-	-
methomyl	0.05	-	-	-	*0.01*	-	-	-	-	-	-	-	-	-	-	-	-
oxamyl	0.05	-	-	*0.01*	*0.01*	-	-	-	-	-	-	**0.1**	-	-	-	-	-
propoxur	0.05	-	-	*0.03*	*0.01*	**0.08**	*0.014*	*0.03*	*0.02*	-	-	*0.049*	*0.02*	**0.09**	*0.02*	*0.02*	*0.01*

[a]: Concentrations printed **in bold** are higher than the limit of quantification; concentrations printed *in italic* are between the detection limit and the limit of quantification (LoQ); - means that concentrations are lower than the detection limit.

Table 5: Results of the chemical analysis of organophosphate insecticides in the rainwater samples (µg/l) performed by HHR-D[a].

Compound	LoQ	Open sampler								Wet-only sampler							
		HORT				BACK				HORT				BACK			
		05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09[b]
azinphos-ethyl	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	*0.018*
azinphos-methyl	0.07	-	-	-	-	-	-[c]	*0.04*	-	-	-	-	-	-	*	*0.05*	*0.04*
bromophos-ethyl	0.03	*0.01*	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
bromophos-methyl	0.02	-	-	*0.015*	-	-	-	-	-	-	-	-	-	-	*	-	**0.021**
chlorfenvinphos	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
demeton-S-methyl	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
diazinon	0.01	-	*0.005*	**0.016**	*0.006*	-	-	*0.004*	-	-	*0.004*	*0.006*	-	-	*	-	**0.021**
dichlorvos	0.03	*0.023*	**0.031**	**0.23**	**0.031**	-	*0.012*	*0.013*	-	*0.012*	-	**0.32**	**0.049**	-	*	-	*0.01*
disulfoton	0.01	*0.003*	**0.028**	**0.017**	*0.004*	-	**0.11**	**0.011**	*0.004*	-	**0.026**	*0.003*	*0.003*	-	*	**0.01**	*0.003*
fenthion	0.007	-	*0.002*	-	-	-	-	-	-	-	-	**0.017**	*0.002*	-	*	-	**0.023**
heptenophos	0.02	-	*0.008*	-	-	-	-	*0.017*	-	-	-	-	-	-	*	-	*0.008*
malathion	0.01	-	-	*0.006*	*0.004*	-	**0.14**	*0.005*	-	-	*0.005*	*0.005*	*0.005*	-	*	-	*0.01*
mevinphos	0.07	-	-	**0.07**	-	-	-	-	-	-	-	-	-	-	*	-	-
parathion-ethyl	0.01	*0.005*	*0.007*	**0.015**	**0.01**	-	**0.062**	*0.006*	*0.003*	*0.004*	*0.006*	*0.006*	**0.012**	*0.003*	*	-	**0.045**
parathion-methyl	0.01	-	-	-	-	-	**0.092**	-	-	-	-	-	-	-	*	-	-
pirimiphos-methyl	0.03	-	-	*0.02*	**0.07**	-	-	-	**0.09**	-	-	**0.08**	*0.01*	-	*	-	*0.01*
pyrazophos	0.03	-	-[d]	-	-	-	-[e]	-	-	-	*0.02*	-	-	-	*	-	*0.02*
tetrachlorvinfos	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	*0.02*
tolclofos-methyl	0.01	*0.007*	**0.01**	-	*0.003*	-	**0.012**	*0.004*	*0.003*	*0.004*	*0.008*	-	-	-	*	-	-
triazophos	0.02	-	-	**0.021**	**0.1**	-	**0.054**	**0.067**	**0.072**	-	-	**0.042**	**0.057**	-	*	**0.079**	**0.091**

[a]: Concentrations printed **in bold** are higher than the limit of quantification (LoQ); concentrations printed *in italic* are between the detection limit and the limit of quantification; - means that concentrations are lower than the detection limit. * means that insufficient rainwater was collected for analysis.
[b]: The LoQ of all organophosphates for the wet-only sample of BACK-07/09 is two times as high as normal due to bad recovery of the internal standard.
[c]: The LoQ of azinphos-methyl for the open sample of BACK-06/11 is 0.7 µg/l due to an interfering peak.
[d]: The LoQ of pyrazophos for the open sample of HORT-06/11 is 0.3 µg/l due to an interfering peak.
[e]: The LoQ of pryazophos for the open sample of BACK-06/11 is 0.7 µg/l due to an interfering peak.

Table 6: Results of the chemical analysis of N-, P-, Cl-containing pesticides in the rainwater samples (µg/l) performed by ZHEW[a].

Compound	LoQ	Open sampler								Wet-only sampler							
		HORT				BACK				HORT				BACK			
		05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09
atrazine	0.1	-	**0.18**	**0.15**	-	-	**0.85**	**0.19**	-	-	**0.16**	**0.15**	-	-	**0.59**	**0.12**	-
azinphos-ethyl	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
azinphos-methyl	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
bupirimate	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
chlorpropham	0.1	**0.13**	-	-	-	*0.06*	-	-	-	**0.14**	-	-	-	*0.06*	-	-	-
chlorothalonil	0.07	-	*0.04*	-	-	-	-	-	-	-	*0.06*	**0.12**	*0.04*	*0.04*	-	*0.04*	-
chloridazon	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
deltamethrin	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
diazinon	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
dichlobenil	0.03	*0.02*	-	*0.02*	-	-	-	-	-	*0.02*	-	-	-	*0.02*	-	*0.01*	-
dichlorvos	0.03	*0.02*	*0.026*	**0.2**	-	-	-	-	**0.04**	*0.02*	-	**0.14**	**0.04**	-	-	*0.02*	-
dimethoate	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
etridiazole	0.15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fenthion	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
heptenophos	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
malathion	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mevinphos (cis & trans)	0.1	-	-	-	-	-	-	-	-	-	-	*0.04*	-	-	-	-	-
parathion-ethyl	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
parathion-methyl	0.1	-	-	-	-	-	-	-	-	-	-	*0.04*	-	-	-	-	-
pirimicarb	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pirimiphos-methyl	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
phosphamidon-trans	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pyrazophos	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
simazine	0.1	-	*0.09*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tolclofos-methyl	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

[a]: Concentrations printed **in bold** are higher than the limit of quantification; concentrations printed *in italic* are between the detection limit and the limit of quantification (LoQ); - means that concentrations are lower than the detection limit.

Table 7: Results of the chemical analysis of organochlorine pesticides and some PCBs in the rainwater samples (µg/l) performed by HHR-D[a].

Compound	LoQ	Open sampler								Wet-only sampler							
		HORT				BACK				HORT				BACK			
		05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09
aldrin	0.02	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
dichlobenil	0.007	**0.02**	-	**0.016**	**0.019**	**0.015**	-	**0.014**	**0.016**	**0.024**	*	**0.014**	**0.023**	**0.016**	*	**0.013**	**0.014**
dieldrin	0.01	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
endosulfan-sulphate	0.007	*0.003*	*0.004*	*0.005*	-	-	*0.004*	0.004	-	*0.004*	*	*0.003*	*0.003*	-	*	*0.003*	-
endrin	0.01	-	-	*0.005*	-	-	-	-	-	-	*	-	-	*0.004*	*	-	-
heptachlor-epoxide A	0.007	-	-	**0.011**	*0.002*	-	-	0.005	-	-	*	-	-	-	*	-	*0.002*
heptachlor	0.007	-	-	-	-	-	*0.002*	-	-	-	*	-	-	-	*	-	-
hexachlorobenzene	0.007	-	*0.003*	-	-	-	-	-	-	-	*	-	-	-	*	-	*0.002*
hexachlorobutadiene	0.007	*0.003*	*0.004*	*0.002*	-	*0.004*	-	-	-	*0.003*	*	-	-	*0.006*	*	-	-
iprodione	0.03	**0.095**	-	**0.085**	**0.093**	-	-	-	**0.049**	**0.096**	*	**0.14**	*0.014*	-	*	**0.093**	*0.008*
procymidone	0.03	-	-	-	-	*0.02*	**0.23**	-	**0.07**	-	*	-	-	-	*	**0.13**	*0.02*
telodrin=isobenzan	0.003	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
vinclozolin	0.01	-	-	**0.028**	*0.004*	*0.006*	-	**0.018**	-	*0.004*	*	**0.02**	-	-	*	**0.021**	-
α-endosulfan	0.02	-	*0.011*	-	-	-	*0.006*	-	-	-	*	-	-	*0.007*	*	-	-
α-HCH	0.02	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
β-endosulfan	0.01	*0.006*	*0.011*	**0.033**	*0.008*	-	*0.01*	**0.026**	*0.008*	*0.012*	*	-	**0.018**	*0.009*	*	*0.009*	*0.011*
β-HCH	0.02	-	*0.008*	-	-	-	*0.01*	-	-	-	*	-	-	-	*	*0.007*	-
δ-HCH	0.003	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
γ-HCH	0.01	**0.056**	-	-	*0.012*	**0.06**	-	**0.016**	*0.007*	**0.056**	*	-	*0.012*	**0.062**	*	-	*0.007*
o,p-DDD	0.007	*0.003*	-	-	-	-	-	-	-	-	*	-	*0.003*	*0.002*	*	-	-
o,p-DDE	0.003	-	*0.002*	**0.031**	**0.004**	-	**0.004**	*0.003*	**0.004**	-	*	*0.002*	**0.01**	**0.006**	*	*0.002*	*0.002*
o,p-DDT	0.03	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
p,p-DDD	0.01	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
p,p-DDE	0.01	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
p,p-DDT	0.01	-	*0.004*	-	-	-	-	-	-	-	*	-	-	-	*	-	-
PCB-028	0.03	-	-	-	-	-	-	*0.02*	-	-	*	-	-	-	*	*0.01*	-
PCB-052	0.03	-	-	**0.04**	*0.01*	-	-	-	-	-	*	-	-	-	*	-	-
PCB-101	0.03	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
PCB-118	0.03	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
PCB-138	0.03	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
PCB-153	0.03	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
PCB-180	0.03	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-
PCB-Balschmitter	0.23	-	-	-	-	-	-	-	-	-	*	-	-	-	*	-	-

[a]: Concentrations printed **in bold** are higher than the limit of quantification (LoQ); concentrations printed *in italic* are between the detection limit and the limit of quantification; - means that concentrations are lower than the detection limit. * means that insufficient rainwater was collected for analysis.

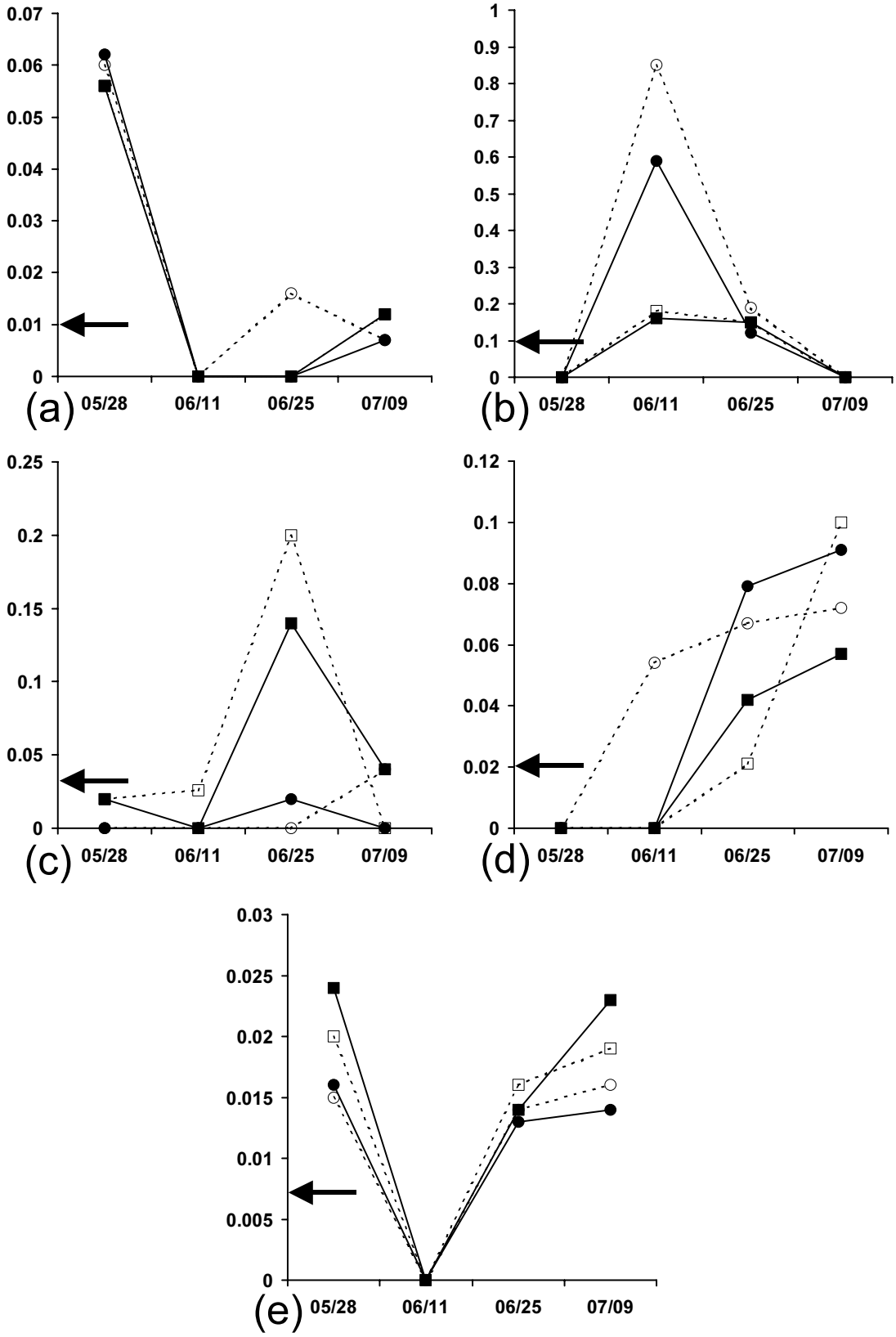

Figure 2: Concentrations in rainwater (µg/l) of pesticides with a typical time pattern during the sampling season at locations HORT (■) and BACK (●). **(a)** γ-HCH (lindane), **(b)** atrazine, **(c)** dichlorvos (ZHEW), **(d)** triazophos, **(e)** dichlobenil (HHR-D). Open markers refer to open samplers and filled markers to wet-only samplers. The arrow indicates the limit of quantification. Dates on the x-axis refer to the final sampling day of the 14-days lasting sampling period.

By far the highest rainwater pollution with pesticides was found in the third sampling period (06/25). In HORT, very high concentrations were found of the OP insecticide dichlorvos (Figure 2C) and of the CARB insecticides and acaricides methiocarb and carbofuran (measured as its metabolite 3-hydroxycarbofuran), irrespective of the sampling method. In addition, on both sampling locations peak concentrations were found of the carboxamide vinchlozonil (fungicide). Finally, for two OC pesticides peak concentrations were found only in the open samplers, but not in the wet-only samplers. This is the case for o,p-DDE in HORT and for beta-endosulfan (insecticide) on both locations.

Concentrations of the OP insecticide and acaricide triazophos increased during the 8 weeks of sampling (Figure 2D), reaching maximum levels in the fourth sampling period (07/09) on both locations. The OC dichlobenil (nitrile herbicide; Figure 2E), the carboxamide iprodione (fungicide), and the OP insecticide diazinon were found regularly during all 4 sampling periods but did not show a peak concentration in a certain sampling period.

In the parallel chemical analyses on samples from open and wet-only samplers, 127 times a compound was found in the open samples and 123 times in the wet only samples. In 84 of these cases, the same compound was found in the parallel samples. For these 84 couples of concentrations, a correlation coefficient of 0.95 was found between open samplers and from wet only samplers, whereas the concentrations in open samplers were significantly higher (t-test, α=0.10) than in wet-only samples. Consequently, in 43 cases a compound was found in the open sampler, which was not found in the parallel wet-only sample, and in 39 cases this was the other way around. When these observations are included, no significant differences are found between concentrations in both samplers (non-parametrical sign-test, α=0.10).

Esterase inhibition assay

Inhibition of bee esterase activity showed a clear dose-response relationship with increasing concentrations of the acetone and the combination fraction of the extract. Up to concentrations equivalent to 25 ml rainwater per ml of final volume in the 96-well plate the inhibition was well described by Michaelis-Menten kinetics of reversible inhibition (Figure 3). Based on the estimated Ki-value for dichlorvos of 1.35 µg/l (95%-confidence interval 1.00-1.79), the Ki-values of all fractions of rainwater extracts (Table 8) were expressed in dichlorvos equivalent (DEQ) concentrations.

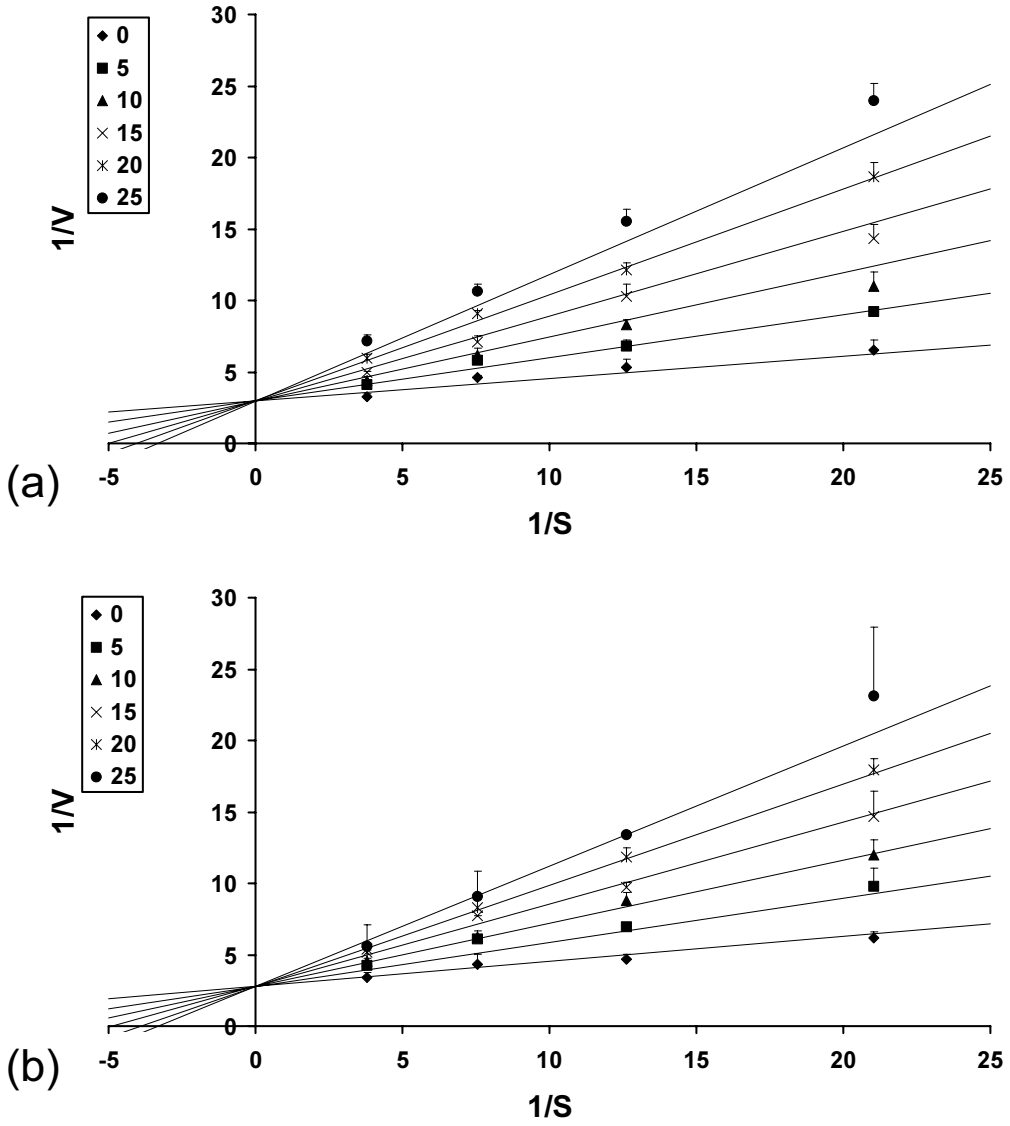

Figure 3: Lineweaver-Burk plot of the reciprocal initial reaction rate (1/V; [s^{-1}]) *vs.* the reciprocal substrate concentration (1/S; [$\mu M]^{-1}$) for **(a)** the combination fraction and **(b)** the acetone fraction of the rainwater extracts of sample BACK-07/09. Rainwater concentrations are expressed as concentration factors of the sample in the microtiter 96-wells plate (see legend). Error bars indicate standard deviation.

The esterase inhibiting potency of the rainwater sample HORT-06/25 was significantly higher than of any other sample (Figure 4), whereas this sample contained also by far the highest toxic potency when the chemical analyses of the rainwater samples were expressed as DEQ concentrations based on honeybee LD_{50}-values. Overall, the correlation between the bio-assay responses of the acetone fraction (open sampler) and the chemical analyses of the rainwater samples collected by the open samplers and the wet-only samplers was 0.83 and 0.86, respectively (Figure 4). Furthermore, the esterase inhibiting potency of the acetone

fraction was significantly greater than of the dichloromethane and hexane fraction (Figure 4). Except for sample HORT-05/28, no significant differences were found between the toxic potency of the combination fraction and the acetone fraction, implying that the dichloromethane and hexane fraction contained no compounds capable of affecting the esterase inhibiting potency of the pesticides eluted by the acetone fraction.

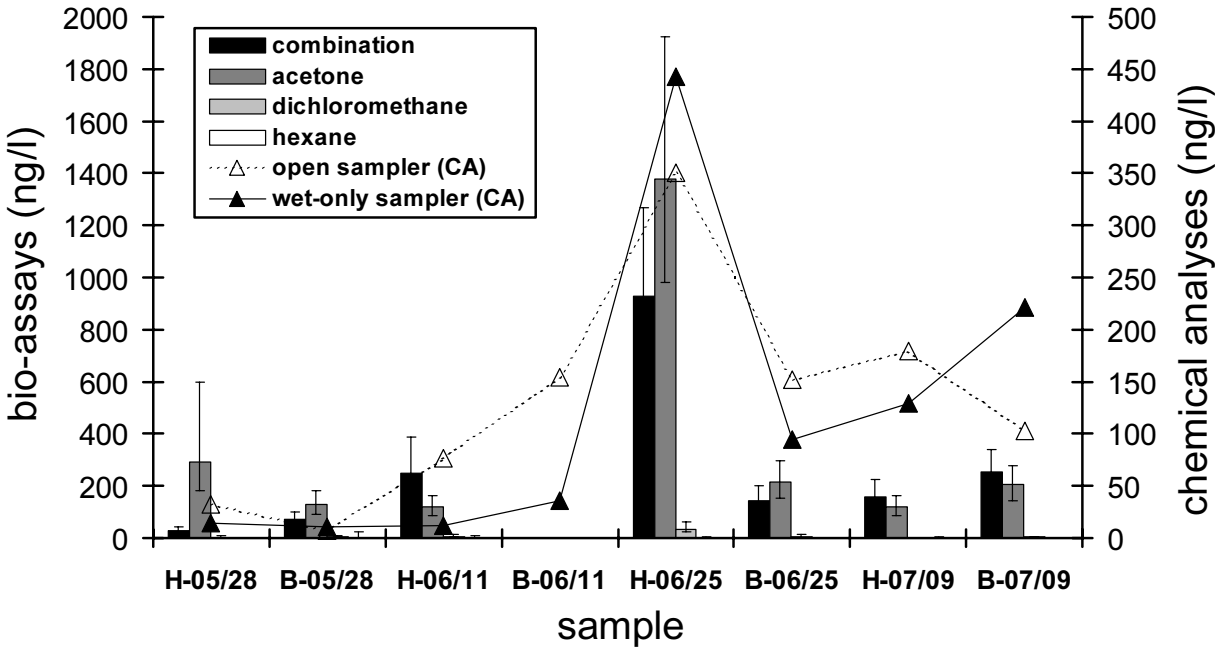

Figure 4: Toxic potency (with 95% confidence interval) of esterase-inhibitors expressed as ng/l dichlorvos-equivalents. Bio-assay equivalents were recalculated from K_i-values of rainwater extracts and dichlorvos. Chemical equivalents were recalculated from concentrations of organophosphate and carbamate insecticides by means of topical LD_{50}-values for honeybees (*A. mellifera*). **CA:** chemical analysis, **H:** HORT, **B:** BACK.

Bioluminescence assay

Especially for the acetone and combination fractions, bioluminescence of the *V. fischeri* bacteria diminished with increased concentrations of rainwater extracts, whereas no toxicity was found when pure and diluted rainwater samples were tested. The lowest observed effect concentrations (LOECs) of the rainwater extracts, expressed as concentration factor in the microtiter well before injection of the bacteria, are given in Table 9. The highest toxicity was found in the same polluted sample of HORT-06/25. A similarly small LOEC was observed for the combination fraction of the parallel sample BACK-06/25. Furthermore, the samples of 07/09 from both locations also showed an increased toxic potency, even in the dichloromethane fraction of HORT.

Table 8: Ki-values (expressed as concentration factor in the well) determined in the esterase inhibition assay for the different fractions of the rainwater samples[a].

fraction of the extract	HORT				BACK			
	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09
combination	49	5.45	1.45	8.48	18.6	*	9.48	5.34
	(33.2-71.0)	(3.60-7.73)	(1.14-1.85)	(6.21-11.2)	(14.4-23.6)		(7.01-12.5)	(4.29-6.47)
acetone	4.58	11.4	0.975	11.3	10.5	*	6.29	6.62
	(2.29-7.07)	(8.68-14.9)	(0.739-1.26)	(8.99-14.4)	(7.80-13.6)		(4.77-8.03)	(5.15-8.26)
dichloromethane	605	296	38.5	-	212	*	418	354
	(293-2.54*10^3)	(133-685)	(22.3-56.0)		(134-326)		(197-1.63*10^3)	(219-608)
hexane	-	1.03*10^3	903	858	2.16*10^3	*	2.62*10^3	5.67*10^3
		(465-6.94*10^3)	(511-2.20*10^3)	(501-1.89*10^3)	(994-66.8*10^3)		(#)	(#)

[a]: 95%-confidence limits given in parentheses; *: insufficient rainfall for performing an esterase inhibition assay; -: no inhibition was found; (#): confidence interval too large to be calculated.

Table 9: Lowest Observed Effect Concentrations (LOECs; expressed as concentration factor in the well before injection of the bacteria) in the bioluminescence assay with *V. fischeri* bacteria for the different fractions of the rainwater samples.

Fraction	HORT				BACK			
	05/28	06/11	06/25	07/09	05/28	06/11	06/25	07/09
combination	31.6	31.6	3.16	10	31.6	31.6	3.16	10
acetone	31.6	31.6	10	31.6	31.6	31.6	31.6	31.6
dichloromethane	>100	100	100	10	100	100	31.6	100
hexane	>100	100	100	>100	31.6	31.6	100	100

Discussion

The goal of the current paper was to compare the integrated toxic potency of rainwater extracts to the chemically analyzed concentrations of the individual pesticides in rainwater. Good correlation was found between the *in vitro* esterase inhibiting potency of rainwater extracts and the chemically analyzed concentrations of OP and CARB insecticides expressed as dichlorvos equivalent (DEQ) concentrations (r=0.83-0.86). Highest esterase inhibiting potency was found in the acetone fraction of the rainwater extract of HORT-06/25, being equivalent to a concentration of 1380 ng dichlorvos per liter rainwater (Figure 4). The high bio-assay response to this sample coincides with relatively high concentrations of the individually analyzed OP compounds dichlorvos, mevinphos and pirimiphos-methyl and the CARB compounds methiocarb and (3-hydroxy-)carbofuran (Tables 4-6), resulting in a calculated DEQ concentration of 351 and 443 ng/l for the open and wet-only sampler, respectively.

So far no environmental criteria have been set for pesticide concentrations in rainwater Therefore, we compared the DEQ concentrations measured in the esterase inhibition assay to critical concentrations of dichlorvos for surface water. The maximum esterase inhibiting potency of 1380 ng DEQ/l exceeded the Maximum Permissible Concentration (MPC) set for dichlorvos in Dutch surface waters (0.7 ng/l; Crommentuijn *et al.*, 1997) almost by a factor 2000, whereas it exceeded the EC_{50} for Daphnia (190 ng/l; Tomlin, 1994) by a factor 7. These results indicate that esterase inhibitors in rainwater are actually toxic to aquatic species and pose a risk for aquatic ecosystems.

With respect to the individual pesticides, analyzed concentrations in the polluted sample HORT-06/25 (wet-only) exceeded the Dutch MPCs for surface water (Crommentuijn *et al.*, 1997; De Bruijn *et al.*, 1999) by a factor 457 for dichlorvos, 25 for mevinphos, 4.9 for propoxur, 3.2 for parathion-ethyl, and 1.3 for triazophos and endrin. The maximum concentrations of the OP compounds dichlorvos and pirimiphos-methyl exceeded the Daphnia

EC_{50} by a factor 1.7 and 1.1, respectively, whereas the maximum concentrations of the OP compounds azinphos-ethyl (11×), azinphos-methyl (22×), mevinphos (21×), parathion-ethyl (40×), pyrazophos (18×) and triazophos (30×), and the CARB insecticide methiocarb (32×) were ≤40 times smaller than the EC_{50} for Daphnia (Tomlin, 1994).

The calculated DEQ concentration based on concentrations of individual OP and CARB insecticides and the topical LD_{50}'s of the individual compounds to *A. mellifera* was on average about 50% of the DEQ concentration determined in the bio-assay. This difference can be attributed to esterase inhibitors that were not chemically analyzed, to inhibitors that still contributed to the total esterase inhibition but were present at concentrations below the detection limit, and to analyzed compounds for which no honeybee LD_{50}-values were available. This underlines the additional value of bioassays to chemical analyses.

In addition, the *in vivo* LD_{50} value of an insecticide is not necessarily proportionally related to its esterase inhibiting potency measured *in vitro*. *In vivo*, differences exist between compounds in uptake and toxicokinetics and insecticides may be activated or degraded. Finally, the adopted concept of concentration addition (CA) based on *in vivo* LD_{50} values has not been proven to be valid for the joint toxicity of mixtures of OP and CARB insecticides, although they are both known to have a similar mode of action *in vivo* (*i.e.* esterase inhibition). However, based on a literature review (Deneer, 2000) it has been concluded that CA is a useful concept to describe the joint toxicity of pesticides to aquatic organisms *in vivo*, although toxicity higher than CA has incidentally been described for aquatic species (Deneer, 2000) and for honeybees (Thompson, 1996) exposed to combinations of an OP insecticide together with another OP or CARB insecticide. For the *in vitro* situation, data from our laboratory (not shown) indicate that the combined esterase inhibiting potency of OP and CARB pesticides can adequately be described by the CA concept. Therefore, it would be best to base DEQ calculations on *in vitro* relative potencies of individually analyzed compounds, rather than based on *in vivo* toxicity data.

The pesticides that possibly cause the increased esterase inhibiting potency of the sample HORT-06/25 (dichlorvos, mevinphos, pirimiphos-methyl, methiocarb and [3-hydroxy-] carbofuran) have not been analyzed in any of the 28 European studies on pesticides in rainwater reviewed by Dubus *et al.* (2000). Of the 66 pesticides (parent compounds and metabolites) analyzed in the present study, only 28 have been analyzed in European rainwater between 1990 and 1997. Of these 28 compounds, 23 have actually been detected in rainwater in Europe (Dubus *et al.*, 2000), whereas 19 of them were found in the present study. Most of

the overlapping fifteen compounds that were detected in both studies had comparable (DDE, hexachlorobenzene, simazine) or lower maximum concentrations in the present study than in the other European studies (Table 10). For the frequently reported pesticides, only the maximum concentration of the herbicide atrazine (850 ng/l) was relatively high in our study, whereas the insecticide propoxur and the fungicide vinclozolin were found more frequently and at relatively high concentrations in our study, possibly because these compounds are mainly used in typically Dutch horticulture under glass. As no study performed in The Netherlands was included in the review by Dubus *et al.* (2000), the pesticide concentrations found in the present study were further compared to year-round monitoring concentrations in 1991-1992 performed by the Province of South-Holland (1994) in rainwater from 4 locations, including HORT (Table 10). Of the overlapping 40 pesticides analyzed in both studies, 13 (mainly OC pesticides) were no longer detected in HORT-samples in 1997, whereas maximum concentrations of another 19 pesticides had decreased. It should be noted, however, that in the 1992 study rainwater was sampled for 52 weeks compared to only 8 weeks in the present study. Therefore, peaks of high pesticide use were possibly not included in the present study. Although a maximum reduction factor of 6.4 was found for vinclozolin at location HORT (Table 10), deposition of this compound is still relatively high in 1997 compared to other European sites. Unfortunately, propoxur and atrazine have not been analyzed in 1992. Only for iprodion (8.8×), pirimiphos-methyl (6.1×), disulfoton (2.8×), beta-endosulphan (2.2×), mevinphos (1.8×) and triazophos (1.1×), higher maximum concentrations were found in our study compared to 1992.

As expected, rainwater from HORT contained more pesticides than from BACK, with an absolute peak of esterase-inhibiting capacity at 06/25 (Figure 4). In general, pesticides were detected in larger numbers and in higher concentrations at HORT than at BACK (Tables 4-7). Nevertheless, some pesticides were consistently found at higher concentrations at BACK than at HORT, both in the open and the wet-only samples. This holds especially for atrazine, procymidon, and triazophos and to a lesser extent for propoxur (Tables 4-7). In The Netherlands, the use of atrazine was only allowed in corn culturing at the time of sampling. As corn is not commonly cultured around location BACK and the other pesticides are frequently used in many other vegetable, fruit and flower cultures, it is impossible to trace the main agricultural source of these pesticides in this relatively background location.

Table 10: Maximum concentrations (ng/l) in rainwater found in this study compared to the distribution of maximum concentrations reported (MCR) for various European sites as reviewed by Dubus *et al.* (2000)[a].

Pesticide	Dubus *et al.*, 2000						This study		Province of South-Holland, 1994 (*n*=4)	
	n	minimum	25th percentile	median	75th percentile	maximum	maximum (*n*=2)	Compared to Dubus *et al.*., 2000	maximum (*n*=4)	Maximum (HORT)
atrazine	26	10	83	135	408	5000	850	>	*	*
DDD	4	66	70.5	93	2651	3500	3	<<	18	3
DDE	6	1	1.7	14.5	95.3	96	31	≈	7	7
DDT	6	2	9.5	72	3044	6000	4	<	*	*
diazinon	4	80	89.3	153	289	322	21	<<	70	40
hexachlorobenzene	5	1	1.5	4.5	183.5	350	3	≈	15	11
lindane	23	6	73	270	550	833	62	<	240	20
parathion-ethyl	6	50	140	200	382	569	62	<	190	50
simazine	13	40	66.5	140	435	8100	90	≈	*	*
carbaryl	1	-	-	110	-	-	45	<	*	*
chlorothalonil	1	-	-	1100	-	-	120	<	*	*
dichlobenil	1	-	-	3120	-	-	20	<	68	63
patrathion-methyl	1	-	-	3400	-	-	92	<	*	*
propoxur	2	27	-	29	-	31	90	>>	*	*
vinclozolin	3	11	-	11	-	16	28	>>	260	180

[a]: The dashed line separates compounds that were reported on ≥ 4 sites (enabling calculation of distribution characteristics) from compounds that were reported on <4 sites. **n**: number of sampling sites for which positive detections were reported. >>: higher than the highest MCR, >: higher than 75% of MCR, ≈: in between 25th and 75th percentile of MCR, <: lower than 25% of MCR, <<: lower than the lowest MCR, -: n<4, no percentiles could be calculated, *: not analyzed.

In the bioluminescence assay for general toxicity, the acetone fraction of the HORT-06/25 extract also yielded a maximum response (Table 9). As *V. fischeri* respond not only to pesticides but also to many other compounds (Kaiser and Palabrica, 1991), the general toxicity of the rainwater extracts is not necessarily caused by pesticides. The relative high toxic potency of the combination fractions of BACK-06/25, HORT-07/09 and BACK-07/09, for example, could therefore not be explained by the analyzed pesticides alone, and is probably caused by other non-volatile organic compounds present in the rainwater.

Rainwater samples were collected by two generally applied methods, both using a dark bottle and a funnel. For both sampler types, (bio-)degradation and evaporation of pesticides can not be completely excluded during the 14-day collecting period. The slightly higher pesticide concentrations in the open samplers are probably due to extra collection of dry deposition or spray drift. Pesticide concentrations in the open samplers correlated very well (r=0.95) with concentrations in the wet-only samplers, indicating that samples collected by both methods are comparable with respect to the composition of the pesticide mix. This makes both methods seem suitable to assess the calculated integrated toxic potency of a rainwater sample.

This study compared the chemically *vs.* biologically determined toxic potencies of pesticides in rainwater. For hazard characterization procedures, it was demonstrated that measuring the integrated toxic potency using bio-assays is a valuable contribution to chemical analyses, in which some contributing compounds are missed because they are not analyzed or present at levels smaller than the detection limit, or because no toxicity data are available for these compounds. The bio-assays can now be integrated in hazard characterization procedures, both as prescreen and for quantification of the total toxic potency. The next step would be the biological validation of the assay. One possibility is the performance of laboratory tests exposing relevant test organisms to extracts of rainwater to determine the consequences of a relevant toxic exposure for the *in vivo* situation. Another approach is measuring the esterase activity in organisms (*e.g.* honeybees) exposed *in situ*, either actively or passively.

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Chapter 5

Estrogenic and esterase-inhibiting potency in rainwater in relation to pesticide concentrations, sampling season and location

Submitted to Environmental Pollution

Timo Hamers[1]
Paul J van den Brink[2]
Lizzy Mos[1]
Sander C van der Linden[1]
Juliette Legler[13]
Jan H Koeman[1]
Albertinka J Murk[1]

Rainwater sampling sites in the Province of South-Holland: site BACK (top) at the shore of Lake Nieuwkoopse Plassen in Noorden, site BULB (middle) in the so-called bulb-area near Hillegom, and site HORT (bottom) in the so-called Westland area with intense greenhouse horticulture in Naaldwijk. Each picture shows the open samplers in the foreground and the wet-only sampler in the background.

[1]: Wageningen University - Toxicology Group; [2]: Wageningen UR - Alterra Green World Research; [3]: Institute for Environmental Studies (IVM) - Vrije Universiteit Amsterdam

Abstract

In a year-round monitoring program (1998), pesticide composition and toxic potency of the mix of pollutants present in rainwater were measured. The goal of the study was to relate atmospheric deposition of toxic potency and pesticide composition to each other and to sampling time and local agricultural activity. Rainwater was collected in 26 consecutive periods of 14 days in a background location (BACK) and in two locations representative for different agricultural practices, *i.e.* intensive greenhouse horticulture (HORT) and flower bulb culture (BULB). Samples were chemically analyzed for carbamate (CARB), organophosphate (OP) and organochlorine (OC) pesticides and metabolites. Esterase inhibiting potency of rainwater extracts was measured in a specially developed bio-assay with honeybee esterases, and estrogenic potency of the extracts was measured in the ER-CALUX reporter gene assay. Multivariate Principal Component Analysis (PCA) techniques proved to be valuable tools to analyze the numerous pesticide concentrations in relation to toxic potency, sampling location, and sampling season. Pesticide composition in rainwater depended much more on sampling season than on sampling location, but differences between SPRING and SUMMER were mainly attributed to local differences in agricultural practice. On average, the esterase inhibiting potency (expressed as dichlorvos equivalent concentrations) exceeded the maximum permissible concentration set for dichlorvos in The Netherlands, and was significantly higher in HORT than in BACK and BULB. Esterase inhibition correlated significantly with OP and CARB concentrations, as expected given the working mechanism of these insecticides. The estrogenic potency incidentally exceeded NOEC levels reported for aquatic organisms and was highest in SPRING. Although estrogenic potency of rainwater correlated with OC concentrations, the ER-CALUX responses could not be attributed to any particular pesticides. Besides, the contribution of non-analyzed xeno-estrogens as alkylphenol(-ethoxylates) and bisphenol-A to the estrogenic potency of rainwater could not be excluded. Further research should focus on the chemical identification of estrogenic compounds in rainwater. In addition, more attention should be given to the ecological consequences of atmospheric deposition of individual pesticides and of total toxic potencies that regularly exceed environmental criteria for Dutch surface waters and/or toxic threshold values for aquatic organisms.

Introduction

Over the past decade, many European and American studies demonstrated deposition of pesticides from the atmosphere (Dubus *et al.*, 2000; Van Dijk and Guicherit, 1999). Evaporation during application or from soil and plant surfaces after application is the main route for pesticides to enter the atmosphere, but spray drift and wind erosion are also important processes. Airborne pesticides may remain in gaseous phase or bind to airborne particulate matter (APM), dependent on their vapor pressure and on APM concentrations. Removal from the atmosphere takes place by dry deposition (*i.e.* gas exchange or APM deposition) or by wet deposition (*i.e.* scavenging from the atmosphere). Complex mixtures of pesticides deposited from the atmosphere may originate from local sources, but also from (long-range) atmospheric transport. Atmospheric deposition of pesticides was demonstrated in relatively background areas, located from 15 km up to ± 1000 km from their possible site of application (Hüskes and Levsen, 1997; Zabik and Seiber, 1993; Biddleman *et al.*, 1993), and may therefore considerably contribute to the environmental loading with pesticides of relative remote areas, which are not directly exposed to direct pesticide use or drift.

In rainwater collected in The Netherlands, average concentrations of some pesticides exceeded the Maximum Permissible Concentration (MPC) set for Dutch surface water by a factor 2-50 (Province of South-Holland, 1994), and peak concentrations of dichlorvos and pirimiphos-methyl even exceeded EC_{50}-values for Daphnids (Chapter 4). Still, comparing concentrations of individual compounds to critical concentrations as MPCs or EC_{50}'s does not account for non-analyzed pesticides or their metabolites, or for the combinatory effect of the mixture of compounds present in the rainwater. Recently, a successful approach was proposed in which small-volume bio-assays were used to assess the integrated toxic potency of all constituents present in the complex mixture of rainwater pollutants (Chapter 4). Although deposition of some individual compounds can directly be attributed to typical temporary and/or local use, little is known so far about the relationship between toxic potency and season or location.

In the present paper, the presence, concentration, deposition, chemical composition, and toxic potency were measured of the mix of pesticides in rainwater in a year-round monitoring program. The goal of the study was to relate atmospheric deposition of toxic potency and pesticides to sampling time and (local) agricultural activity. In addition, an assessment was made of the contribution of particular (groups of) compounds to the toxic potency of the

rainwater. Wet deposition was selected, because wet scavenging form the gas-phase is an important deposition route at land surfaces, especially for gaseous pesticides that are soluble in water (Van Pul *et al.*, 1999).

During 26 periods of 14 days, rainwater samples were collected at three sampling sites in the Province of South-Holland. Two sites were situated in areas with specific agricultural activities (*i.e.* greenhouse horticulture [HORT] and flower bulb culture [BULB]). The third site was chosen to represent a relative background location for The Netherlands (BACK).

Samples were chemically analyzed for 11 carbamate (CARB), 22 organophosphate (OP) and 25 organochlorine (OC) pesticides or their metabolites and an additional number of 7 polychlorinated biphenyls (PCBs). The integrated toxic potency of the samples was biologically analyzed in two small-scale bio-assays, which respond to compounds with a specific and known mode of action:

1. The esterase inhibition assay responds specifically to OP and CARB insecticides. The toxicity of these insecticides is based on their ability to block esterases, thus inhibiting the hydrolysis of the neurotransmitter acetylcholine by acetylcholinesterase (AChE). The assay was specifically optimized for analyzing rainwater samples (Chapter 3) and good correspondence was found between the response of the esterase assay and chemical analyzed OP and CARB levels in a pilot study (Chapter 4). The response of the assay has been recalculated into equivalent rainwater concentrations of dichlorvos, which is a very potent esterase inhibitor.
2. The ER-CALUX assay responds specifically to compounds, which activate the estrogen receptor (ER) and thus can mimic the activity of female sex hormone estradiol. The bio-assay was developed by Legler *et al.* (1999) and has now been applied for the first time to measure estrogenic potency in rainwater samples. ER-CALUX responses were recalculated into equivalent rainwater concentrations of estradiol. Little is known about the estrogenic potency in rainwater, but *in vitro* estrogenic activity has mainly been described for OC and pyrethroid pesticides that may be found in rainwater (*e.g.* Safe, 2001; Metzler and Pfeiffer, 2001; Go *et al.*, 1999).

Because this study encompasses the measurements of many variables (pesticides), it is difficult to grasp the general pattern of (cor)relations among pesticide concentrations and their relationship with season, location and bio-assay responses. For this dimension reduction has to be carried out with the use of multivariate techniques (see Sparks *et al.*, 1999 for a review on their use in ecotoxicology). In this paper the multivariate method Principal

Component Analysis in combination with Monte Carlo permutation test (Ter Braak, 1995) was used to obtain this dimension reduction and the significance of indicated differences between seasons and locations.

Material and Methods

Sampling sites

Rainwater was collected at three sites in the Province of South Holland:

1. Site BACK is regarded to be a relatively unpolluted area for the Dutch situation. The samplers were situated at the shore of Lake Nieuwkoopse Plassen in Noorden.
2. Site HORT is characterized by intensive greenhouse horticulture in the so-called Westland area. The samplers were situated at the Research Station for Floriculture and Glasshouse Vegetables in Naaldwijk.
3. Site BULB is characterized by flower bulb culture in the so-called Bulb-area. The samplers were situated in the middle of bulb fields just outside the city of Hillegom.

Rainwater sampling

At all three sampling sites, rainwater was sampled using two different methods. For chemical analysis, samples were collected in wet-only collectors, which are open only during rainfall. Because wet-only samples were too small to allow also for bio-assay analysis, additional samples were collected in larger open collectors. Earlier results had shown that bio-assay results did not differ between samples from open and wet-only samplers (Chapter 4).

Each site had 10 open samplers consisting of a clean amber bottle (2.5 l) placed at 1.5 m above ground level. Each bottle was topped with a weathered polyester funnel (452 cm^2). Samplers were covered during pesticide applications at the sampling site itself (site BULB). Wet-only sampling was performed with one REG-100 sampler (Van Essen, Delft) per sampling site, consisting of a glass bottle (5 l) connected to a glass funnel (400 cm^2). Both bottle and funnel were placed in a dark container with a lid, which opened via a rain sensor connected to the lid. Samples were collected from January 7, 1998 to January 6, 1999 in 26 consecutive 14-day sampling periods (Table 1).

Upon arrival at the laboratory, pH and volume of each sample were measured and samples were stored in the dark at 4°C. The next day, each wet-only sample was divided into unequal sub-samples needed for the four different chemical analyses, depending on the sample volume

the required volume per analysis (see below), and the priority given to each analysis, which decreased in the order CARB, OP, OC and N-,P-,Cl-containing compounds. From each open sample, a maximum of 9 liter was extracted for bio-assay analysis (see below).

Chemical analyses

Four types of chemical analyses were performed, each aiming at a specific group of compounds (Tables 2-5), *i.e.* (1) organophosphate (OP) insecticides, (2) N-methyl carbamate (CARB) insecticides, (3) organochlorine (OC) pesticides, and (4) some additional N- and Cl-containing OC herbicides and fungicides. The methods of analysis have previously been described in detail in Chapter 4.

Briefly, OP and OC insecticides were analyzed by the Laboratory of Polder Board Delfland (HHRD, Delft). Analysis of OP insecticides was based on a method described in VPR-C88-18 (1988). Prefiltered subsamples of 1000 ml of rainwater were extracted on a solid phase extraction (SPE) C18 column, with subsequent acetone elution. The extract was dried with Na_2SO_4 and evaporated to a final volume of 1 ml. The OP insecticides were detected by GC with a flameless N,P detector.

The analysis of OC insecticides was based on a gaschromatographic method described by the Netherlands Standardization Institute in protocols NEN-5734 (1995) and NEN-6406 (1990). Subsamples of 250 ml of rainwater were extracted by liquid-liquid extraction with hexane. The extract was dried with Na_2SO_4 and evaporated to a final volume of 0.5-1.0 ml. Samples were divided in two parts, which were led over columns of different polarities. After elution, detection was performed by Electron Capture Detection.

Analyses of CARB pesticides and N-, P- or Cl-containing compounds were performed by the Central Laboratory of Water Authority Hollandse Eilanden & Waarden (ZHEW, Rotterdam). CARB compounds were extracted from 4 ml subsamples of acidified rainwater that were filtered over a polystyrene divinyl benzene SPE-cartridge. Compounds were eluted, separated on a second C18 column and hydrolyzed to methylamines on a third Aminex column. The methylamines were transformed into an iso-indol, which was detected fluorimetrically (NEN-6403; 1997).

Analysis of N-, and Cl-containing OC herbicides and fungicides was based on a protocol that was especially developed by ZHEW. After addition of 25 g NaCl to 250 ml subsample, liquid-liquid extraction was performed with ethylacetate. The extract was dried with Na_2SO_4

and evaporated to a final volume of 0.5 ml. The compounds were detected by GC-MS (Chapter 4).

Sample preparation for bio-assays

Prefiltered (sea sand; Merck) subsamples of a maximum of 9000 ml rainwater were extracted on a SPE column (2000 mg C18: JT Baker), which was eluted with nanograde acetone (Prochem). Extracts were evaporated under a gentle N_2-flow at 30°C and dissolved in a final volume of 25 µl 1-propanol (99+% Acros). After performing the esterase inhibition assay, the remaining 1-propanol was evaporated and the extract was dissolved in 50 µl of DMSO for determination of the estrogenic potency.

Esterase inhibition assay

The esterase inhibition assay is based on inhibition of esterases obtained from heads of working honey bees (*Apis mellifera*) by organophosphate and carbamate insecticides, thus preventing the hydrolysis of the substrate N-methylindoxylacetate (MIA: 1-H-indol-3-ol, 1 methyl-acetate [ester]; Sigma) into acetate and the green fluorescent N-methylindoxyl. MIA was kept in a stock solution of 1 mg/ml (5.3 mM) in methanol (HPLC Labscan) at -20°C in the dark. The assay itself was performed in KP_i-buffer (0.1 M; pH=7.0). Test conditions and way of application on rainwater extracts have been described in detail in Chapters 3 and 4, respectively). The esterase inhibiting potency of each rainwater sample can be described by Michaelis-Menten kinetics for reversible inhibition, resulting in an inhibitor constant K_i. The assay response was calculated into equivalent concentrations of the standard OP compound dichlorvos by dividing the K_i-value of dichlorvos by the K_i-value of the rainwater sample (see Chapters 3 and 4). Dichlorvos (2,2-dichlorovinyldimethyl-phosphat; Riedel-De Haën) was kept in stock solutions in methanol (HPLC Labscan) at 4°C in the dark.

ER-CALUX

The estrogenic potency of the rainwater samples was determined in the Estrogen Receptor (ER)-mediated Chemical Activated LUciferase gene eXpression (ER-CALUX) assay, earlier described in detail by Legler *et al.* (1999). The assay makes use of a T47D cell line that is stably transfected with a plasmid for the luciferase gene under control of three estrogen responsive element (pEREtata-Luc). Briefly, T47D.luc cells were plated in a 96 wells plate (Nuclon, Denmark) at a density of 5000 cells in 0.1 ml DMEM F12 medium without phenol

red (Gibco) with 5% (dextran-coated charcoal) stripped serum per well and 1% non-essential amino acids (Gibco). After 24 h of incubation (37°C, 5% CO_2) the medium was refreshed, and cells were incubated for another 24 h. The medium was removed and cells were exposed to medium containing rainwater extracts in DMSO (0.2% at maximum). After 24 h of exposure, medium was removed and cells were lysed on ice in 30 µl low salt buffer (10 mM Tris, 2 mM DTT, 2mM 1,2-diaminocyclohexane-N,N-N'-N'-tetraacetic acid, pH=7.8). The plate was frozen at –80°C for at least 30 min and measured within one week. Before measurement, plates were thawed at room temperature on a shaker for 20 min. Cell lysate was transferred to a white, non-transparent 96-wells plate (Costar). Luciferase production was determined well by well in a luminometer (Labsystems Luminoskan RS) by (1) injecting 100 µl of luciferin containing flashmix (20mM tricine, 1mM $[MgCO_3]_4Mg[OH_2]5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2 mM DTT, 470 µM luciferin, 5 mM ATP; pH=7.8), (2) measuring light production for 2 s, and (3) extinguishing the signal by injecting 50 µl of a 0.2M NaOH solution.

Rainwater extracts were tested in triplicate per 96-wells microtiter plate. The estrogenic potency of the samples was recalculated into estradiol (E2) equivalent concentrations by interpolating the ER-CALUX response (induction factor) in a simultaneously tested calibration curve of E2 (cumulative fit; $r^2 \geq 0.98$; SlideWrite 4.0 for Windows). Quantification into E2 equivalent (EEQ) concentrations was only allowed for ER-CALUX responses between the quantification limit (0.5 pM) and the EC_{50} (6 pM) (Legler *et al.*, 2002b; Murk *et al.*, 2002). Preliminary range-finding experiments were performed to determine dilution factors for the rainwater samples to meet this criterion.

Data-analysis

Multivariate techniques were used for dimension reduction, *i.e.* to reduce the complex pesticide data set into a two-dimensional summary (ordination diagram). In this way a graphical summary of the data set was obtained, providing an overview of mutual relationships between pesticide concentrations on the one hand and their relation with season, sampling locations and bioassay responses on the other hand. As ordination technique, Principal Component Analysis (PCA) was used in combination with Monte Carlo permutation tests (Ter Braak, 1995). PCA is based on a linear response model similar to the linear model underlying regression analysis, but explanatory variables are not measured (manifest) but latent. In PCA, imaginary latent explanatory variables are calculated from the data set which

best explain the variation in pesticide concentrations between samples. The first latent variable is constructed in such a way that it explains the largest part of the total variance possible, the second one the largest part of the remaining variance, etc. After extracting more and more latent variables, PCA eventually accounts for all the variance of a data set (Ter Braak, 1995). The first two latent variables are normally used as axes to construct an ordination diagram and the weights of the pesticides and samples with these variables are plotted in the diagram represented by points. After construction of the diagram, several sample characteristics can be superimposed on the diagram, *i.e.* they can be regressed on the axes using the sample points. Sampling SEASON and LOCATION are characteristics that can be used as explanatory variables, explaining the arrangement of the samples and pesticides as given by PCA. Similarly, the bio-assay responses can be superposed on the diagram as characteristics of the samples and pesticides arranged by PCA. These sample characteristics do not play an active role within PCA analysis, *i.e.* they are simply regressed on the latent variables after analysis has taken place.

Four different principal component analyses (PCA1 through PCA4) were performed, *i.e.* the analysis of the relationship between

1. concentrations of all pesticides and the explanatory variables season and location,
2. total deposition of all pesticides and the explanatory variables season and location,
3. OP and CARB pesticide concentrations and the bioassay responses (sample characteristics)
4. OC pesticide concentrations and the bioassay responses (sample characteristics).

Pesticide depositions (ng/m^2) for PCA2 were calculated by multiplying analyzed concentrations (ng/l) with rainfall ($mm=l/m^2$) during each sampling period. Bio-assay responses and pesticide concentrations were analyzed separately for OP and CARB compounds (PCA3) and OC compounds (PCA4), because the bio-assays were to respond differently to the different groups of pesticides.

For each PCA, different data sets were used. Out of a total number of 75 rainwater samples available for chemical analyses, PCA1 and PCA2 were performed on only those 43 samples for which sufficient rainwater was collected to analyze all compounds. Similarly, in the other PCAs only 55 and 46 samples were used for which sufficient rainwater was collected to analyze OP and CARB compounds (PCA3) or OC compounds (PCA4).

To test the significance of the differences in pesticide composition and concentrations between for instance samples taken in SPRING or SUMMER, Redundancy Analysis (RDA) was

performed, followed by Monte Carlo permutation. RDA is the constrained form of PCA. In RDA explanatory variables (or sample characteristics) do play an active role in contrast to the situation in PCA. RDA constrains the ordination to that part of the total variance of a data set that is explained by a given set of explanatory variables (*e.g.* SEASON and LOCATION). In this way differences in pesticide concentration (or deposition) between explanatory variables are maximized. Furthermore, the fraction of the total variance explained by the explanatory variables is calculated in RDA. Differences in pesticide composition and concentrations among seasons were tested using nominal variables denoting SEASON as explanatory variables and nominal variables denoting LOCATION as covariables. Differences among locations were tested by introducing LOCATION as explanatory variable and SEASON as covariable.

In all analyses, pesticide concentrations or depositions were square root-transformed and centered to down-weight high values and to make their means mathematically equally important. Bio-assay responses (expressed as equivalent concentrations of dichlorvos or estradiol) were also square-root transformed prior to applying them as sample characteristics in PCA3 and PCA4. The analyses were performed in the computer program Canoco for Windows 4 (Ter Braak and Smilauer, 1998). In case a pesticide was not detected in a given rainwater sample, half of the detection limit (PCA1, PCA3, PCA4) or half of the lowest calculated deposition over the 26 sampling periods (PCA2) was used. Pesticides that were not detected in any of the rainwater samples were omitted from PCA analysis.

Finally, a two-way ANOVA (SPSS 10.1) was used to determine the effect of the variables LOCATION and SEASON on square-root transformed bio-assay results. Differences among locations and seasons were analyzed using Tukey's test, in case variances between groups were equal or Dunnett's T3 test in case homogeneity of variances was violated.

Results

Rainfall and rainwater sampling

Rainfall was extremely high in 1998 (Table 1): annual totals ranged from 1168 (BACK) to 1285 mm (HORT) and were 50% higher than the Dutch long-time average value of 803 mm (KNMI, 1999). In periods 18 (September) and 22 (November), more than 100 mm had fallen within the 14-day sampling period at all 3 sampling sites. Rainfall was highest in SUMMER and

lowest in SPRING and decreased between locations in the order HORT >BULB≈ BACK, though no significant differences were found between sampling seasons or sampling sites.

Of the 26 sampling periods, only one period (16; August) experienced no rain, so that 25 open and 25 wet-only rainwater samples were collected at each sampling site. Unfortunately, the open rainwater samples of period 23 could not be extracted for bio-assay analyses, due to logistical problems. Thus, a total number of 25 CARB analyses (requiring only 4 ml of rainwater sample) and 24 bio-assay analyses were performed for each sampling site (Tables 3 and 6, respectively). Numbers of OP (Table 2) and OC analyses (Tables 4 and 5) were smaller, due to larger sample volumes needed for analyses.

Table 1: Rainfall (mm) at the 3 sampling sites during the 14-days lasting sampling periods in 1998.

Number	Sampling period	Season	Sampling site		
			BACK	HORT	BULB
1	7 January – 21 January	WINTER	33	50	40
2	21 January – 4 February	WINTER	4	5	5
3	4 February – 18 February	WINTER	2	3	3
4	18 February – 4 March	WINTER	32	51	32
5	4 March – 18 March	WINTER	84	78	76
6	18 March – 1 April	SPRING	6	11	8
7	1 April – 15 April	SPRING	55	82	61
8	15 April – 29 April	SPRING	42	40	40
9	29 April – 13 May	SPRING	4	28	33
10	13 May – 27 May	SPRING	23	15	11
11	27 May – 10 June	SPRING	89	60	61
12	10 June – 24 June	SUMMER	59	74	71
13	24 June – 8 July	SUMMER	40	40	43
14	8 July – 22 July	SUMMER	35	50	30
15	22 July – 5 August	SUMMER	35	24	40
16	5 August – 19 August	SUMMER	0	0	0
17	19 August – 2 September	SUMMER	78	86	84
18	2 September – 16 September	SUMMER	136	125	139
19	16 September – 30 September	AUTUMN	20	12	15
20	30 September – 14 October	AUTUMN	50	55	54
21	14 October – 28 October	AUTUMN	81	71	65
22	28 October – 11 November	AUTUMN	121	140	116
23	11 November – 25 November	AUTUMN	21	38	25
24	25 November – 9 December	AUTUMN	28	37	26
25	9 December – 23 December	WINTER	48	53	52
26	23 December – 6 January	WINTER	42	57	43
Σ(1-26)	7 January 1998 – 6 January 1999		1168	1285	1173

Chemical analyses

All OP compounds analyzed were detected in any of the 55 samples analyzed (Table 2). Tolclofos-methyl, dichlorvos, pirimiphos-methyl, heptenophos, diazinon and disulfoton were most abundant. Out of the 10 CARB insecticides analyzed, 7 were actually detected in one or

more of the 75 samples analyzed, with propoxur and methiocarb found most often (Table 3). Only half of the OC compounds analyzed have actually been found in rainwater (Tables 4 and 5). γ-HCH (lindane) and hexachlorobutadiene were detected very regularly, and dichlobenil even almost year-round.

Table 2: Presence, average concentration (ng/l) and maximum concentration (ng/l) of OP insecticides analyzed in rainwater[a].

Compound	DL	Number of times detected			Average concentration			Maximum concentration		
		BACK (n=18)	HORT (n=18)	BULB (n=19)	BACK	HORT	BULB	BACK	HORT	BULB
azinphos-ethyl	9	2	1	0	-	-	-	64	30	-
azinphos-methyl	20	2	1	0	-	-	-	100	190	-
bromophos-ethyl	9	2	1	1	-	-	-	19	12	12
bromophos-methyl	5	0	1	0	-	-	-	-	26	-
chlorfenvinphos	8	1	2	0	-	-	-	14	370	-
demeton-*S*-methyl	20	1	0	2	-	-	-	30	-	160
diazinon	4	7	4	3	4.2	4.3	4.4	16	22	36
dichlorvos	9	7	11	6	9.1	9.3	10.2	77	300	81
disulfoton	3	3	5	5	7.9	8.0	8.1	800	95	1100
fenthion	2	3	2	1	2.0	-	-	9	7	15
heptenophos	7	4	6	10	5.8	5.9	6.1	62	26	120
malathion	4	2	1	1	-	-	-	15	44	18
mevinphos	20	4	3	2	13.4	13.8	-	60	70	40
parathion-ethyl	3	3	7	6	1.8	1.9	1.9	15	29	10
parathion-methyl	3	1	0	1	-	-	-	12	-	24
pirimiphos-methyl	10	5	5	10	9.6	9.8	10.0	40	30	60
pyrazofos	10	3	1	2	8.5	-	-	70	50	50
tetrachlorvinfos	10	2	0	1	-	-	-	40	-	30
tolclofos-methyl	3	4	10	13	3.6	3.7	3.8	20	31	41
triazofos	6	2	0	0	-	-	-	48	-	-

[a]: Average concentrations are based on square-root transformed data, which have been weighed for rainfall and then been back-transformed. In these calculations, half of the detection limit (DL (ng/l)) has been filled out for samples <DL. Average concentrations were only calculated for compounds with n>2.

Table 3: Presence, average concentration (ng/l) and maximum concentration (ng/l) of CARB insecticides analyzed in rainwater[a].

Compound	DL	Number of times detected			Average concentration			Maximum concentration		
		BACK (n=25)	HORT (n=25)	BULB (n=25)	BACK	HORT	BULB	BACK	HORT	BULB
3-hydroxycarbofuran	20	0	2	0	-	-	-	-	100	-
aldicarbsulfon	10	1	0	1	-	-	-	20	-	20
carbaryl	20	0	1	1	-	-	-	-	20	30
carbofuran	10	1	0	0	-	-	-	60	-	-
methiocarb	20	0	8	1	-	19.4	-	-	80	20
methomyl	10	0	7	0	-	15.8	-	-	150	-
propoxur	10	3	5	8	6.4	6.7	14.2	20	30	100
aldicarb	10	0	0	0	-	-	-	-	-	-
aldicarbsulfonoxide	30	0	0	0	-	-	-	-	-	-
oxamyl	10	0	0	0	-	-	-	-	-	-

[a]: Compounds below the dotted line have not been detected. For further explanation, see Table 2.

Table 4: Presence, average concentration (ng/l) and maximum concentration (ng/l) of OC compounds analyzed in rainwater[a].

Compound[b]	DL	Number of times detected			Average concentration			Maximum concentration		
		BACK (*n*=14)	HORT (*n*=19)	BULB (*n*=16)	BACK	HORT	BULB	BACK	HORT	BULB
dichlobenil	2	9	13	10	4.6	5.3	4.8	31	18	19
endosulfansulphate	2	0	1	2	-	-	-	-	3	4
heptachlor-epoxide-A	2	0	4	1	-	1.2	-	-	3	7
heptachlor	2	1	2	1	-	-	-	3	3	2
hexachlorbutadiene	2	5	11	5	2.2	2.5	1.9	9	10	9
procymidone	10	0	1	8	-	-	18.0	-	10	60
telodrin = isobenzan	1	5	4	4	1.0	0.6	0.8	3	8	2
vinclozolin	4	2	6	2	-	3.2	-	16	13	14
α-endosulfan	6	1	2	1	-	-	-	9	10	7
β-endosulfan	4	4	4	3	4.0	3.4	3.2	19	19	17
β-HCH	7	1	0	0	-	-	-	16	-	-
δ-HCH	1	2	2	2	-	-	-	2	2	2
γ-HCH	4	6	11	5	4.2	8.1	5.1	16	38	35
o,p-DDD	2	0	2	0	-	-	-	-	4	-
o,p-DDE	1	2	3	2	-	0.6	-	1	2	2
PCB-052	10	0	1	1	-	-	-	-	10	10
Aldrin	7	0	0	0	-	-	-	-	-	-
dieldrin	4	0	0	0	-	-	-	-	-	-
endrin	4	0	0	0	-	-	-	-	-	-
hexachlorobenzene	2	0	0	0	-	-	-	-	-	-
iprodion	8	0	0	0	-	-	-	-	-	-
o,p-DDT	9	0	0	0	-	-	-	-	-	-
p,p-DDD	3	0	0	0	-	-	-	-	-	-
p,p-DDE	4	0	0	0	-	-	-	-	-	-
p,p-DDT	3	0	0	0	-	-	-	-	-	-
PCB-028	10	0	0	0	-	-	-	-	-	-
PCB-101	10	0	0	0	-	-	-	-	-	-
PCB-118	10	0	0	0	-	-	-	-	-	-
PCB-138	10	0	0	0	-	-	-	-	-	-
PCB-153	10	0	0	0	-	-	-	-	-	-
PCB-180	10	0	0	0	-	-	-	-	-	-

[a]: Compounds below the dotted line have not been detected. For further explanation, see Table 2.

[b]: Identification of OC compounds is solely based on a validated method using gaschromatographic retention times coupled to electron capture detection. As no further mass spectometry was performed, the possibility of false positive analyses cannot be fully excluded.

Table 5: Presence, average concentration (ng/l) and maximum concentration (ng/l) of additional OC herbicides and fungicides analyzed in rainwater[a].

Compound	DL	Number of times detected			Average concentration			Maximum concentration		
		BACK (*n*=19)	HORT (*n*=21)	BULB (*n*=21)	BACK	HORT	BULB	BACK	HORT	BULB
Atrazine	30	4	1	1	19.3	-	-	50	40	70
Chlorpropham	30	2	3	11	-	17.8	139.7	60	140	7400
Chlorthalonil	20	1	2	2	-	-	-	30	80	60
Chloridazon	80	0	0	0	-	-	-	-	-	-
Etridiazole	50	0	0	0	-	-	-	-	-	-
Simazine	40	0	0	0	-	-	-	-	-	-

[a]: Compounds below the dotted line have not been detected. For further explanation, see Table 2.

Some compounds were found more frequently at one location compared to the others. Dichlorvos (Table 2), methiocarb and methomyl (Table 3), and γ-HCH and hexachlorobutadiene (Table 4) were most frequently found at location HORT, whereas heptenophos and pirimiphos-methyl (Table 2), procymidone (Table 4) and especially chlorpropham (Table 5) were most frequently found at location BULB. No such typical compounds were found for location BACK, although diazinon (Table 2) and atrazine (Table 5) were detected relatively more often at BACK than at HORT and BULB.

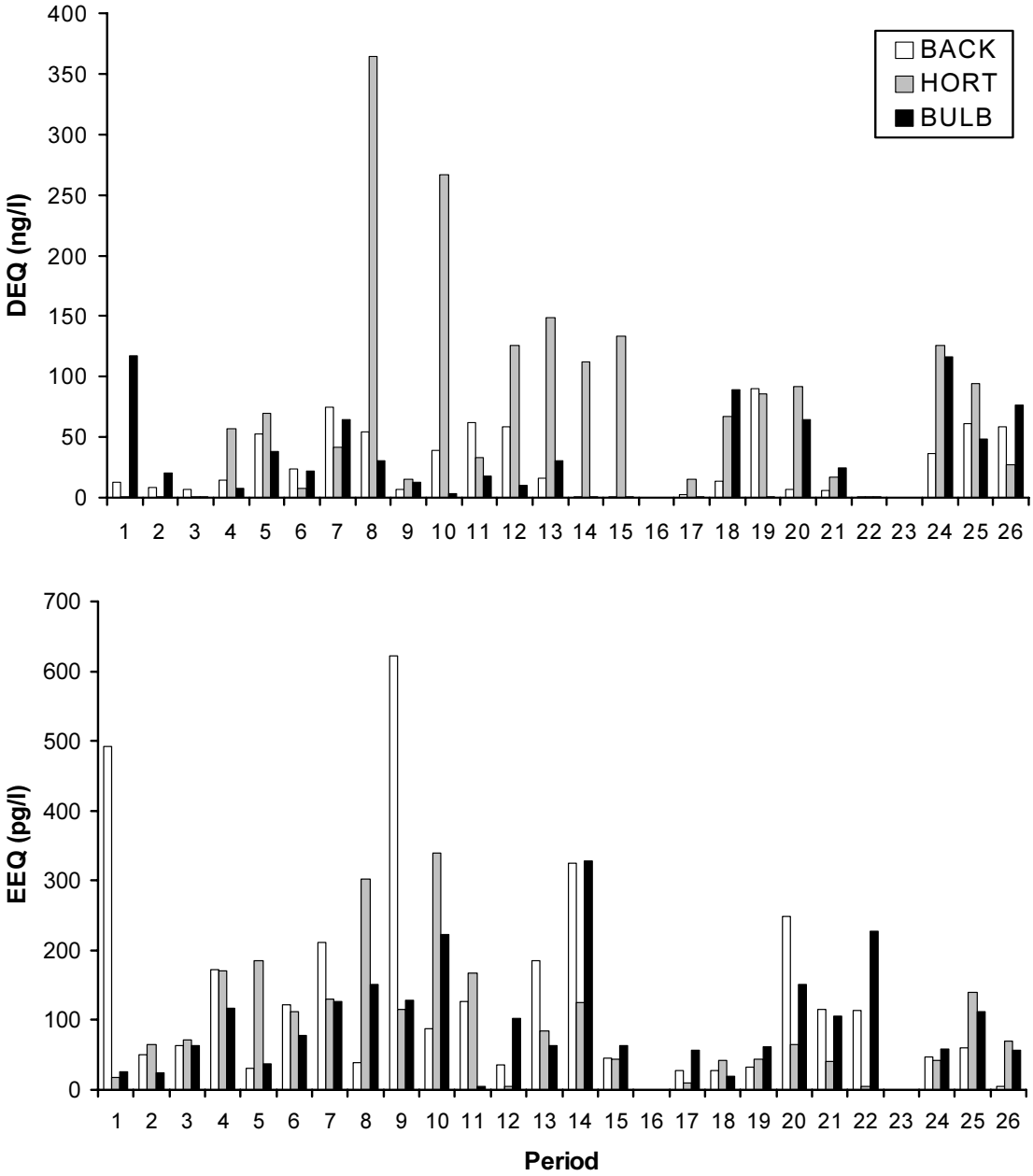

Figure 1: Bio-assay responses to the rainwater extracts collected year-round at locations BACK, HORT and BULB in 1998. Esterase inhibiting potency (top) and estrogenic potency (bottom) are expressed as equivalent concentrations of dichlorvos (DEQ) and estradiol (EEQ), respectively. No rainwater was available for analysis in period 16 (no rainfall) and 23 (logistic problems). Further characteristics of the rainwater samples are given in Table 1.

Bio-assay results

The esterase inhibiting potency and the estrogenic potency of the rainwater is expressed in dichlorvos equivalent (DEQ) or estradiol equivalent (EEQ) concentrations, respectively (Figure 1). Esterase inhibiting potencies differed significantly between locations but not between seasons, with higher DEQ concentrations at location HORT than at BULB and BACK (Tables 6 and 7). Estrogenic potencies differed significantly between seasons but not between locations, with EEQ concentrations in SPRING higher than in SUMMER and WINTER (Tables 6 and 7). These results suggest that different compounds are responsible for the responses in the two assays. This is supported by the fact that no correlation was found between DEQ and EEQ concentrations (r=-0.013).

Table 6: Presence, average concentration and maximum concentration of the dichlorvos equivalent (DEQ) concentration and estradiol equivalent (EEQ) concentration analyzed in rainwater using the esterase inhibition assay and the ER-CALUX assay, respectively[a].

Bio-assay	LQEC	Maximum equivalent concentration			Average equivalent concentration			Number of samples ≥ LQEC		
		BACK	HORT	BULB	BACK	HORT	BULB	BACK (n=24)	HORT (n=24)	BULB (n=24)
DEQ (ng/l)	1.2	90.0	364.4	117.1	29.4	68.2	37.9	21	20	20
EEQ (pg/l)	4.7	621.7	339.5	328.2	107.4	83.6	97.1	23	22	23

[a]: Average concentrations have been weighed for rainfall. In these calculations, half of the lowest quantifiable equivalent concentration (LQEC) was filled out for samples <LQEC.

Table 7: Effect of the explanatory variables SEASON and LOCATION on the bio-assay responses (ANOVA). Differences between variables are tested using post-hoc tests.

ESTERASE INHIBITION ASSAY

Variable	**p-value**	**Multiple comparisons (Dunnett's T3 test)**
SEASON	>0.10	
LOCATION	**0.011**	

	BACK	HORT	BULB
BACK	X		
HORT	**0.053**	X	
BULB	>0.10	**0.080**	X

SEASON*LOCATION	>0.10	

ER-CALUX ASSAY

Variable	**p-value**	**Multiple comparisons (Tukey test)**
SEASON	**0.039**	

	SPRING	SUMMER	AUTUMN	WINTER
SPRING	X			
SUMMER	**0.048**	X		
AUTUMN	>0.10	>0.10	X	
WINTER	**0.098**	>0.10	>0.10	X

LOCATION	>0.10	
SEASON*LOCATION	>0.10	

Principal Component Analysis

The relationship between the concentrations of chemically analyzed pesticides and the explanatory variables sampling SEASON and LOCATION (PCA1) is shown in Figure 2A. For reasons of clarity, the underlying individual samples are not shown in this biplot. Instead, they are summarized in the explanatory variables SEASON and LOCATION. Each open square in Figure 2A indicates the centroid of all samples collected during a specific SEASON or collected at a specific LOCATION. Interpretation of biplots as Figure 2A is illustrated for compounds diazinon and tolclofos-methyl in Figure 2B. The distance of the pesticide point to the origin indicates the relative magnitude of the differences in concentrations, *i.e.* the deviation from an average sample that is located in the origin. For instance, diazinon concentrations vary more from their average concentration than tolclofos-methyl concentrations. Such relative differences do not indicate that absolute concentrations (in terms of µg/l) are also larger for diazinon, because pesticide concentrations were centered prior to PCA analysis. The relative concentration of a pesticide in a particular SEASON or LOCATION can be derived by perpendicularly projecting the 'explanatory variable point' on the ‘pesticide line’ that is drawn through a pesticide point and the origin of the plot. In our example, the explanatory variable SUMMER is projected on the ‘diazinon line’ far away from the origin, but on the same side of the origin as the diazinon point, indicating that relatively high concentrations of diazinon are found in SUMMER. The 'explanatory variable point' WINTER projects on the other side of the origin, compared to the diazinon point, indicating that diazinon concentrations were relatively low in WINTER. The greater the distance between the projection of explanatory variable SUMMER and the origin, the higher the concentration of a pesticide is in this particular season. Diazinon concentrations differ more from average concentrations in SUMMER than tolclofos-methyl concentrations because the projection of the explanatory variable SUMMER on the individual ‘pesticide lines’ is larger for diazinon than for tolclofos-methyl (Figure 2B).

Figure 2A shows that pesticides in rainwater can roughly be divided in three groups, *i.e.* pesticides present in relatively high concentrations during SPRING (right bottom), pesticides present in relatively high concentrations during SUMMER (right top), and pesticides present in relatively low concentrations not directly correlated to any season (scattered around the origin).

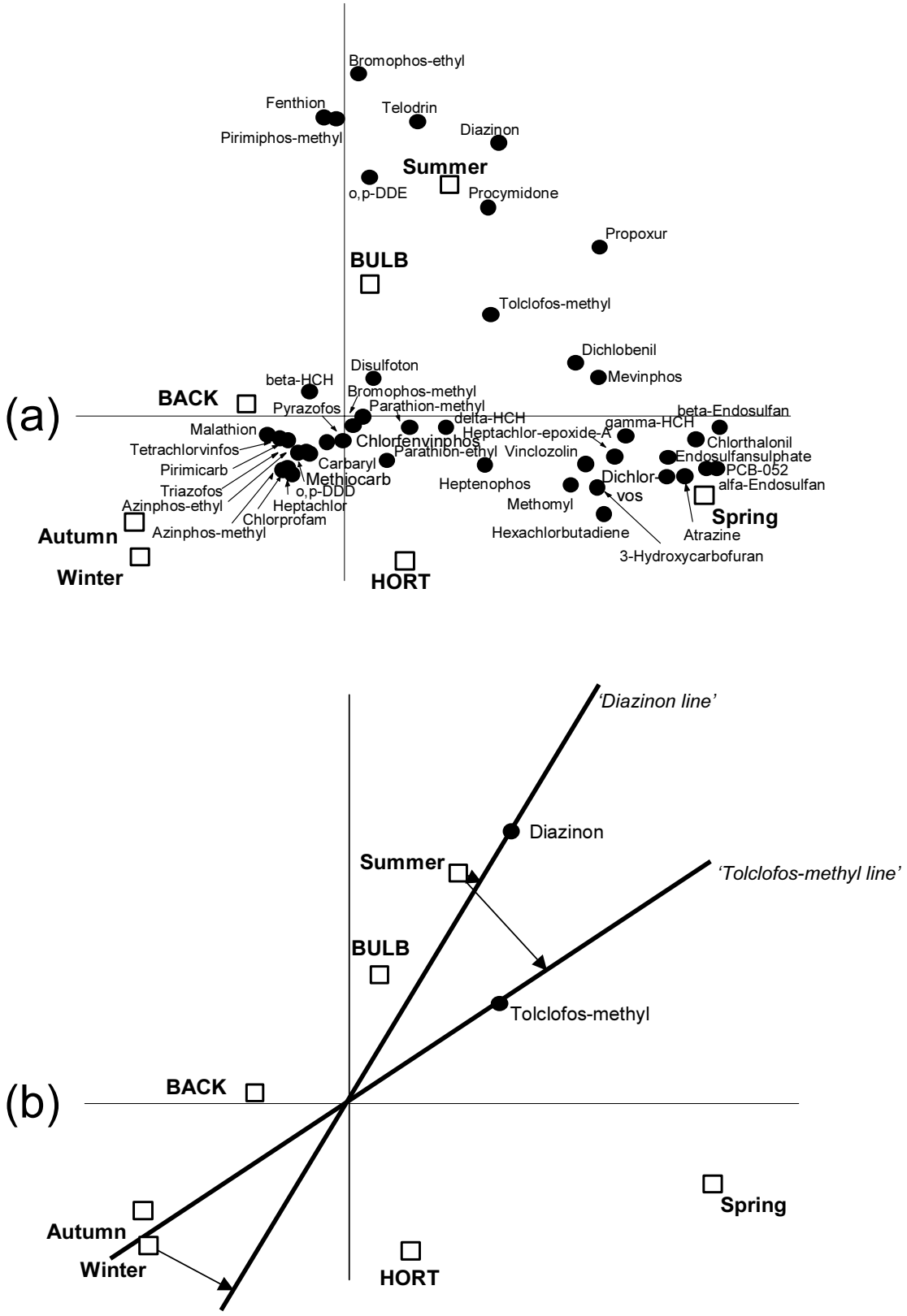

Figure 2: PCA biplot **(a)** showing the relation between sampling season and location and concentrations of 44 pesticides (PCA1). Open squares represent season and location and black dots represent the individual pesticides. For percentages of total variance and results of additional Monte Carlo permutation tests see Tables 8 and 9. Interpretation of the positioning of the pesticides in the biplot relative to the explanatory variables is illustrated for compounds diazinon and tolclofos-methyl **(b)** out of a total of 44 compounds (see text).

Monte Carlo permutation tests indeed indicated that pesticide composition significantly differed between all four sampling seasons (Table 8A). Additionally, significant differences in pesticide composition were found for rainwater samples from locations BULB and HORT (Table 8B), as can also be seen from the relatively large distances between both locations in Figure 2. The results of PCA2 on pesticide depositions (ng/m^2) and the explanatory variables SEASON and LOCATION were comparable (data not shown), although differences between seasons and especially between locations BULB and HORT were less pronounced than for pesticide concentrations.

Table 8: Results of Monte Carlo permutation tests: significance (p) of the differences between (**a**) sampling SEASON and (**b**) sampling LOCATION with respect to pesticide concentration (Figure 2; PCA1), and (**c**) significance of the relation between bioassay response and pesticide concentrations (Figures 3 and 4; PCA3 and PCA4).

a	SPRING	SUMMER	AUTUMN	WINTER
SPRING	X			
SUMMER	**0.032**	X		
AUTUMN	**0.018**	**0.005**	X	
WINTER	**0.018**	**≤0.001**	**0.015**	X
b	BACK	HORT	BULB	
BACK	X			
HORT	>0.10	X		
BULB	>0.10	**0.042**	X	
c	OP and CARB	OC		
EEQ	>0.10	**0.005**		
DEQ	**0.0033**	>0.10		

In PCA3 and PCA4, not only the relation of pesticide concentrations with nominal explanatory variables SEASON and LOCATION was analyzed, but also with continuous sample characteristics like bioassay responses. As bio-assay results indicated that esterase inhibiting potency and estrogenic potency may be attributed to different sets of compounds, two separate PCAs including bio-assay responses as sample characteristics were run, *i.e.* strictly on OP and CARB compounds (PCA3; Figure 3) and on OC compounds (PCA4; Figure 4). In Figures 3 and 4 the continuous variables are shown with arrows pointing to the underlying individual samples (not shown) with the highest bioassay response. The interpretation of the relationship between bio-assay responses and pesticide concentrations is analogous to the nominal explanatory variables SEASON and LOCATION described above.

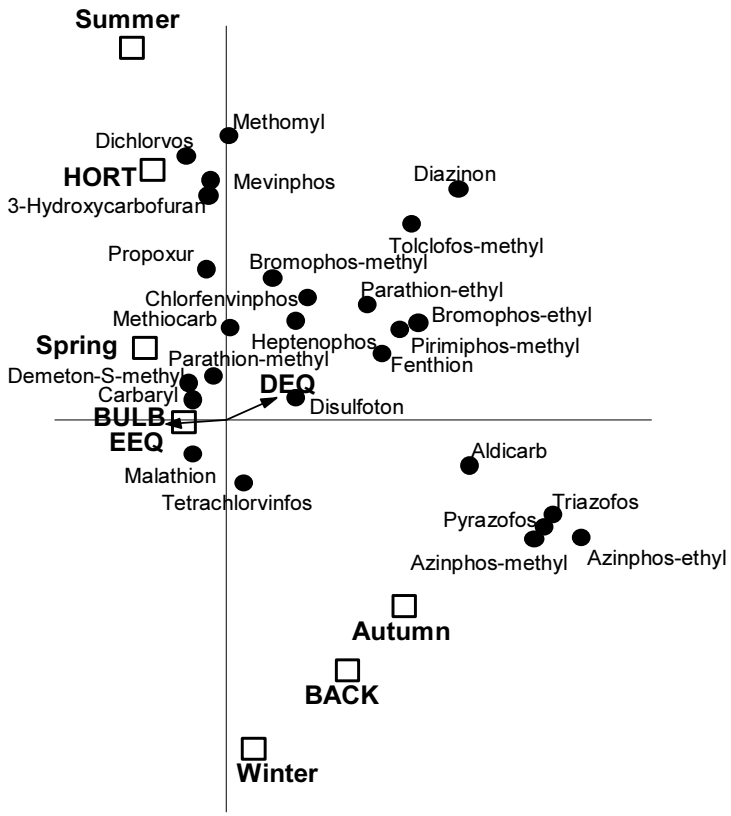

Figure 3: PCA biplot showing the relation between bio-assay responses and the concentrations of OP and CARB pesticides (PCA3). For percentages of total variance and results of additional Monte Carlo permutation tests see Tables 8 and 9.

Concentrations of most OP and CARB compounds correlated with SUMMER and location HORT (Figure 3), located in the top part of the biplot diagram. In addition, a small group of compounds (located under the first principal axis) correlated with AUTUMN and WINTER and location BACK. Differences between sampling seasons and sampling locations were associated more with the vertical secondary axis than with the horizontal first axis (Figure 3; Table 9). Apparently, the horizontal distribution of OP and CARB compounds (especially pyrazofos, triazofos and azinphos-[m]ethyl) is determined not only by the explanatory variables SEASON and LOCATION but also by another (unknown) factor (Figure 3).

Due to the scatter of OP and CARB compounds in Figure 3, the DEQ-arrow representing the esterase inhibition response cannot point into one distinct direction. However, Monte Carlo permutation tests pointed out that the response of the esterase inhibition assay significantly correlated (Table 8C) with OP and CARB concentrations in the rainwater samples, as was to be expected based on the esterase inhibiting working mechanism of these compounds. No such correlation was found for the ER-CALUX responses (Table 8C): the

EEQ arrow points into the direction opposite from where OP and CARB pesticides are plotted (Figure 3), confirming that both bio-assays respond to different compounds.

The arrow representing the estrogenic potency of the rainwater samples (expressed as EEQ) points directly in the 'cloud' of OC compounds that highly correlated to SPRING samples (PCA4; Figure 4). Monte Carlo permutation tests confirmed that estrogenic potency significantly correlated with OC concentrations (Table 8C). This result does not necessarily imply that the compounds plotted in the same direction from the origin as the EEQ arrow were indeed responsible for the estrogenic response in the ER-CALUX assay (see also discussion). The esterase inhibiting potency of the rainwater samples (DEQ) did not correlate at all with OC concentrations (Figure 4; Table 8C).

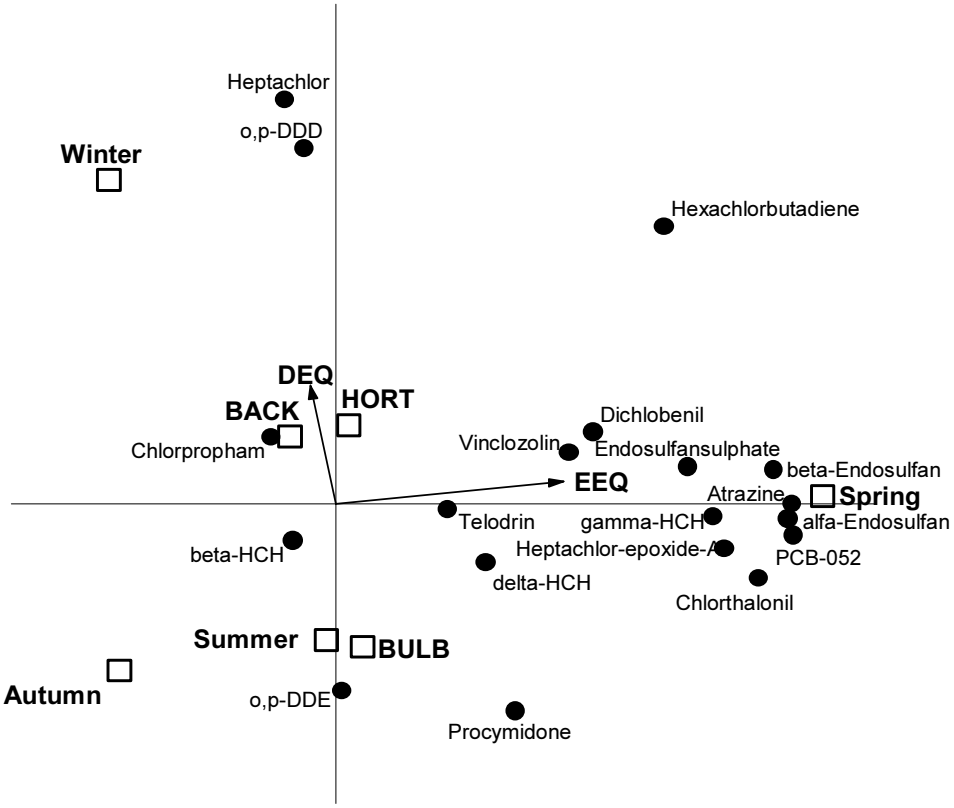

Figure 4: PCA biplot showing the relation between bio-assay responses and the concentrations of OC pesticides (PCA4). For percentages of total variance and results of additional Monte Carlo permutation tests see Tables 8 and 9.

Table 9: Percentages of the total variance that are displayed on the first and second for the four PCA-analyses. The Table also indicates which fraction of the variance is explained by the explanatory variables and which part of this variation is displayed on the first and second axis.

Name	Data set	% of total variance displayed on		% of total variance explained by the explanatory variables and which part is displayed on the axes		
		axis 1	axis 2	% of total variance	of which on axis 1	of which on axis 2
PCA1	all pesticide concentrations	18	9	19	26	11
PCA2	all pesticide depositions	17	11	19	27	8
PCA3	OP and CARB concentrations	15	13	14	4	30
PCA4	OC concentrations	13	10	21	41	9

Discussion

Pesticide composition in rainwater

Differences in pesticide composition in rainwater samples from HORT and BULB (Table 8) reflect the different agricultural practices at both locations, and suggest that atmospheric deposition of pesticides largely depends on local use and subsequent local emission. Rainwater from HORT frequently contained OP and CARB insecticides in relatively high concentrations (dichlorvos, methiocarb, methomyl; Figure 3, Tables 2 and 3). Rainwater from BULB often contained (peak concentrations of) OC compounds such as the pre-emergence herbicide chlorpropham (Table 5) and the fungicide procymidone (Table 4). Indeed, data from Statistics Netherlands (CBS, 2001) on pesticide use in 1998 confirm that these compounds were mainly applied in greenhouse horticulture and flower bulb culture, respectively. Methiocarb and methomyl were mainly used in flower culture under glass (chrysanthemums, roses) and dichlorvos in paprika and cucumber culture under glass. Chlorpropham and procymidone were mainly applied in tulip, lily, narcissus and hyacinth culture, although they were also intensively used in potato (chlorpropham) and onion (both) culturing (CBS, 2001).

At location BULB, (pre-emergence) herbicides were found at the start of the growing season (late WINTER and early SPRING), whereas fungicides were found after flowering in late SPRING and SUMMER. At location HORT, pesticides were mainly found during the growing season (SUMMER). Given the fact that pesticide use is relatively low in both locations outside the growing season (AUTUMN and WINTER), it is obvious that differences in pesticide composition are better explained by the variable SEASON than LOCATION, although the differences are related to local agricultural practices.

Relatively high local use of insecticides at location HORT is also reflected in the esterase inhibition assay, which responds specifically to OP and CARB insecticides. DEQ

concentrations in rainwater were significantly higher at site HORT than at BULB and BACK (Table 7).

When comparing the chemical composition of rainwater in this study to previous studies, it was found that 15 of the 65 compounds analyzed were previously detected in rainwater in any of the European studies reviewed by Dubus *et al.* (2000) and in the pilot study of 1997 (Chapter 4). Results from the pilot study already pointed out that maximum concentrations of most pesticides in Dutch rainwater were (much) lower than maximum concentrations reported in other European studies (Chapter 4). This trend is confirmed by the present study, in which maximum concentrations were within the same order of magnitude or (much) lower than in the pilot study. The maximum concentration measured for atrazine was even 12 times lower than in the pilot of 1997, whereas 3 of the 15 compounds were no longer detected (*i.e.* DDT, hexachlorobenzene and simazine). A similar comparison could be made to an earlier study performed by the Province of South-Holland (1994) on OC and OP compounds in rainwater in 1991-1992. Out of 21 OC compounds analyzed in both studies, 7 were no longer detected in 1998, whereas maximum concentrations in the remaining 14 had declined since 1991-1992. Major decreases were found for vinclozolin (16×), procymidone (7×), δ-HCH (7×), γ-HCH (6×) and iprodion, which was not detected anymore. For vinclozolin and iprodion, concentrations had even dramatically decreased since the pilot study of 1997. These results suggest that national and European restrictions on OC pesticide use have successfully decreased their atmospheric deposition over the past decade.

In contrast, atmospheric deposition of OP pesticides did not show a univocal decrease over the past decade. For 11 of the 18 OP compounds analyzed in both studies, maximum rainwater concentrations were lower in the present study than in 1991-1992. For 4 of the remaining 7 OP pesticides, however, maximum concentrations were considerably higher than in 1991-1992, *i.e.* disulfoton (55×), chlorfenvinphos (9×), demeton-*S*-methyl (8×), and azinphos-methyl (3×). Maximum concentrations of these compounds had even increased since the 1997 pilot study (Chapter 4). Still, average esterase inhibiting potency of the rainwater samples was lower in the present study than in the 1997 pilot study, and the maximum DEQ concentrations had declined by almost a factor 4.

Chemical explanation of the bio-assay responses

The esterase inhibition assay responds specifically to OP and CARB insecticides (Chapter 3). Although relatively high bio-assay responses coincided with relatively high dichlorvos and

mevinphos concentrations, no direct correlations were found between esterase inhibition and the concentration of any individual OP or CARB insecticide over the whole sampling period of 52 weeks. Nevertheless, Monte Carlo permutation tests clearly revealed a clear correlation between the esterase inhibition response and the concentrations of all OP and CARB compounds detected in rainwater (Table 8C).

Estrogenic activity has been described for a much wider variety of chemical structures than esterase inhibition. (Xeno-)estrogens are generally characterized by a ring structure that is hydrolyzed either in the parent compound or after metabolization (Blair *et al.*; 2000). Thus, many compounds with affinity for the estrogen receptor contain phenol groups (Katzenellenbogen, 1995), but exceptions have been described especially for non-phenolic aromatic pesticides, which may however be contaminated by hydrolyzed impurities (Blair *et al.*, 2000). With respect to pesticides, *in vitro* estrogenic activity has mainly been described for (metabolites of) OC compounds such as o,p'-DDT, methoxychlor, kepone, toxaphene, endosulfan, dieldrin, chlordane (*e.g.* reviewed by Safe, 2001; Metzler and Pfeiffer, 2001).

Indeed, ER-CALUX responses of rainwater extracts highly correlated with the presence of OC compounds (Table 8C) deposited in SPRING (Figure 4; Table 7). This does not automatically imply that the ER-CALUX response should be attributed to the compounds actually shown in Figure 4. For instance, atrazine, γ-HCH (lindane), and dichlobenil highly correlated with the ER-CALUX response (Figure 4), but the individual compounds failed to induce ER-mediated reporter gene activity in the ER-CALUX (Legler *et al.*, 2002b; Legler et al., 2002c; and data not shown, respectively). The estrogenic activity of the rainwater samples should therefore be attributed to ER-active compounds that coincide with these compounds, as may be the case with their metabolites. Indeed, ER-agonistic activity has been described for metabolites of γ-HCH (Petit *et al.*, 1997; Flouriot *et al.*, 1995). Endosulfan is the only compound located next to the EEQ arrow (Figure 4) for which weak estrogenic activity has been reported (Petit *et al.*, 1997; Legler *et al.*, 1999). However, applying the molar estrogenic equivalency factor determined in the ER-CALUX assay ($EEF=1*10^{-6}$; Legler *et al.*, 1999), the maximum endosulfan concentration of 19 ng/l (Table 4) only accounts for an EEQ concentration of $13*10^{-3}$ pg/l. As this estrogenic activity is four orders of magnitude lower than the matching EEQ concentrations measured in the rainwater (period 11; Figure 1), it is unlikely that endosulfan is responsible for the estrogenic potency in rainwater.

The ER-CALUX response may also be attributed to ER-agonists that have not been chemically analyzed. These may be active ingredients of pesticides such as methoxychlor and chlordane (Legler *et al.*, 1999), pesticide metabolites or pesticide adjuvants (see below).

Pyrethroid insecticides were not analyzed in the present study but may also be responsible for the ER-CALUX response of the rainwater samples. Recently, contradictive reports have been published regarding the steroidal activity of these compounds. Estrogenic activity of several pyrethroids including permethrin was demonstrated by Go *et al*. (1999). Using ER-regulated recombinant yeast assays, Tyler *et al.* (2000) confirmed that the primary breakdown product of permethrin has ER-agonistic activity, whereas its secondary metabolite has ER-antagonistic activity. Both activities were much higher than the weak estrogenic activity of the parent compound. Interestingly, Tyler *et al.* (2000) found that the estrogenic potency of permethrin from four different suppliers relative to estradiol ranged from 10^{-7} to zero (*i.e.* not estrogenic at all). The authors attributed these differences to different isomeric compositions or to impurities. In three different *in vitro* assays, Saito *et al.* (2000) did not find any (anti)estrogenic effects for eight synthetic pyrethroid insecticides including permethrin. For four pyrethroids tested including permethrin, Sumida *et al.* (2001) also found no ER-mediated luciferase induction in a reporter gene assay irrespective of metabolic treatment (S9-mix).

Pesticide adjuvants are another group of non-analyzed compounds, which may be responsible for the estrogenic potency of the rainwater samples. For instance, nonylphenol-ethoxylates and octylphenol-ethoxylates are surfactants used as wetting agents and emulsifiers in agrochemicals (Renner, 1997). The short-chained breakdown products of the ethoxylates and especially nonylphenol are well-known ER agonists (*e.g.* Petit *et al.*, 1997; Legler *et al.*, 2002b). Although large-scale use of these ethoxylates in cleaning applications has been restricted in Europe since 1995 (Renner, 1997), their estimated use in agrochemicals of 7785 kg in The Netherlands in 1998 (CBS, 2001) may contribute considerably to the estrogenic potency of atmospheric pollution. Within the framework of the National Research on Estrogenic Compounds (LOES), the presence of octylphenol and nonylphenol-ethoxylates has recently been demonstrated in rainwater in concentrations up to 0.28 and 0.99 µg/l, respectively (Vethaak *et al.*, 2002). Applying the highest molar EEF for these compounds (*i.e.* $EEF=2.3*10^{-5}$ for nonylphenol; Legler *et al.*, 2002b), these concentrations can be recalculated into a maximum EEQ concentration of 28.5 pg/l, which is in the same order of magnitude as measured in rainwater in the present study.

Based on the analyses described here, it is impossible to attribute the estrogenic potency of the rainwater extracts to one of the measured compounds. The combination of chemical and bio-assay analyses should be applied more elaborately in a so-called Toxicity Identification and Evaluation (TIE) program so that the estrogenic potency of rainwater can be chemically evaluated. First, active samples are separated into different fractions and tested again in the ER-CALUX assay. Next, fractions with highest estrogenic potency in the assay are chemically analyzed. This is a very difficult step because it comes down to searching for the unknown. Finally, the estrogenic potency of the suspected individual compounds is confirmed or denied in the ER-CALUX. Preliminary TIE-results from rainwater samples additionally collected in 2000 suggest that bisphenol-A (BPA) may be partly responsible for the estrogenic potency of rainwater (data not shown). In ER-active fractions, BPA concentrations ranged from 90 to 450 ng/l rainwater, with highest concentrations in fractions showing highest estrogenic potency. No BPA was detected in inactive fractions. Applying the molar EEF for BPA (EEF=7.8*10^{-6}; Legler *et al.*, 1999), BPA concentrations measured could explain up to 2% of the total estrogenic activity measured in the active rainwater fractions. Within the LOES study (Vethaak *et al.*, 2002), BPA has also been demonstrated in rainwater from a sampling site near the North Sea coast, but concentrations were lower (up to 57 ng/l). BPA is an abundant environmental xeno-estrogen (Krishnan *et al.*; 1993) used for production of polycarbonate plastics and coatings, but there are no records of use of BPA in pesticide formulations.

Ecological relevance of toxic potency in rainwater

Of course, aquatic organisms are not directly exposed to rainwater concentrations, because rainwater pollutants are very diluted when deposited in surface water and will partly adsorb to sediments, suspended matter, and plants. Nevertheless, it is considered to be undesirable that the maximum DEQ concentration of 364 ng/l measured in rainwater (HORT; period 8) exceeded the EC_{50} for *Daphnia* (190 ng/l; Tomlin, 1994) by almost a factor 2. In The Netherlands, no criteria are set for pesticides in rainwater, but average DEQ concentrations (29-68 ng/l; Table 6) exceeded the maximum permissible concentration (MPC) of dichlorvos set for Dutch surface waters (0.7 ng/l; RIVM, 1999) by a factor 42 to 97. MPCs for surface water have been set for 31 of the 58 individual (metabolites of) pesticides analyzed in the present study (RIVM, 1999). For 24 compounds, concentrations higher than MPC were found in one or more of the rainwater samples tested. Concentrations of

dichlorvos, mevinphos, parathion-ethyl and β-endosulfan incidentally even exceeded the MPCs (0.7, 1.6, 1.9 and 0.4 ng/l, respectively) by more than one order of magnitude. Fenthion, heptenophos, mevinphos and o,p-DDE exceeded MPCs in 10-20% of all samples analyzed, β-endosulfan, propoxur and parathion-ethyl in 20-30% and dichlorvos in 44%. In 78% and 45% of samples analyzed, surface water MPCs were exceeded for one or more OP/CARB or OC compounds, respectively. The fact that both the aggregated DEQ concentration and the analyzed concentrations of individual pesticides exceed MPC values for surface water, implies that rainwater pollutants actually need to be diluted or adsorbed after deposition in aquatic ecosystems in order to meet the quality criteria for surface water.

No environmental criteria are available for estradiol (E2) in surface water, but the estrogenic potency in rainwater expressed as EEQ concentrations can be compared to aquatic *in vivo* toxicity data of estrogens, as was recently done by Murk *et al.* (2002). For plasma levels of vitellogenin (VTG), which is an estrogen-inducible yolk precursor protein that is often measured in fish as a biomarker for estrogen exposure, lowest observed effect concentrations (LOEC) in male rainbow trout range from 0.3 ng/l (immature; 28 weeks of exposure; Sheahan *et al.*, 1994) to 10 ng/l (adult; 3 weeks of exposure; Routledge *et al.*, 1998). For decreased egg production in fathead minnow, Kramer *et al.* (1998) calculated an EC_{10} (considered to be equal to the predicted no effect concentration [PNEC]) and an EC_{50} value of 6.6 and 120 ng/l, respectively. The maximum rainwater EEQ concentration of 0.62 ng/l measured in the present study (Table 6) is one order of magnitude lower than this PNEC value, but is two times higher than the LOEC level for VTG induction. The average rainwater EEQ concentration of 0.1 ng/l (Table 6) is equal to the no observed effect concentration (NOEC) for VTG induction reported by Sheahan *et al.* (1994). Although induced vitellogenesis is not an adverse effect as such, it corresponds with decreased egg production in female fish (Kramer *et al.*, 1998). As *in vitro* results may be an underestimation or an overestimation of *in vivo* responses (Routledge *et al.*, 1999; Legler *et al.*, 2002d), the measured EEQ levels in rainwater should be considered undesirable and require further study.

In conclusion, the aggregated DEQ and EEQ concentration measured in the bio-assays together with the analyzed concentrations of individual pesticides demonstrate that rainwater is frequently polluted above threshold level for surface water and incidentally at effect levels for aquatic organisms, also in relatively remote and seemingly protected areas. Because rainwater pollutants are diluted and adsorbed when deposited in surface water, aquatic ecosystems will be exposed to lower concentrations of pesticides and are not expected to be

directly at risk. Possibly, shallow and mainly rainwater-fed water pools are an exception to this rule, and further studies should focus on such pools, especially in remote areas.

Use of multivariate techniques

This is one of the few studies using PCA and Monte Carlo permutation techniques to analyze the relationship between chemical composition of environmental samples on the one hand and nominal explanatory variables (SEASON and LOCATION) or continuous sample characteristics (DEQ or EEQ concentrations) on the other hand (see Sparks *et al.*, 1999 for review). In our opinion, our results demonstrate that PCA biplots enable a transparent projection of numerous data of chemical analyses. The additional value of the explanatory variables SEASON and LOCATION can directly be seen from the plots and is relatively easy to interpret. Moreover, the correlations between esterase inhibition and OP/CARB concentrations and between ER-CALUX responses and OC concentrations (Table 8C) demonstrated the additional value of multivariate analyses, because no such correlations were found between bio-assay responses and the concentration of any individual compound.

Despite the transparency of PCA biplots, the PCA biplot of Figure 2 did not reflect the fact that certain compounds are characteristic for a certain sampling LOCATION as good as the average concentrations represented in Tables 2-5. This can be explained by the fact that PCA requires a complete data set, which implies that PCA1 (Figure 2) is restricted to these samples for which the complete set of four chemical analyses had been performed, whereas all samples that had been analyzed were taken into account to calculate the average concentrations listed in Tables 2-5, *i.e.* also samples that were too small to perform other chemical analyses. Characteristic compounds correlate better with their sampling LOCATION in PCA3 and PCA4, because all OP/CARB analyses were taken along in PCA3 (Figure 3), and all OC analyses in PCA4 (Figure 4), irrespective whether other analyses were performed or not.

Conclusions

In 1998, rainwater in The Netherlands incidentally contained levels of pesticides that were above effect levels for aquatic organisms, although levels were generally lower than in earlier other European (1990-1997) and Dutch studies (1991 and 1997). The total esterase inhibiting potency and estrogenic potency of rainwater pollutants also exceeded environmental criteria

and/or effect levels for *Daphnia* and fish. Further studies should focus on the ecological consequences of rainwater concentrations exceeding toxic thresholds, especially in remote areas. Pesticide composition in rainwater depended more on sampling season than on sampling location. Still, differences between SPRING and SUMMER were mainly attributed to local differences in agricultural practice during the growing seasons, as reflected by differences between locations HORT and BULB. The esterase inhibiting potency of rainwater depended on the sampling location, with highest potency at location HORT, and was very well explained by OP and CARB concentrations in the samples. The estrogenic potency of rainwater was highest in SPRING and correlated very well with the presence of OC compounds in rainwater. It could not be concluded to which pesticide the ER-CALUX response should be attributed. Other compounds that were not analyzed in the present study, such as alkylphenol(-ethoxylates) and bisphenol-A, should not be excluded. These compounds are no active ingredients of pesticides, but their presence may simply coincide with elevated OC concentrations during spring time. Although sampling season and location only explained 14-21% of the total variance in the data set, they are concluded to be meaningful explanatory variables, because Monte Carlo permutation tests pointed out that differences in pesticide concentrations between seasons and locations are not randomly distributed.

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Chapter 6

Lack of a distinct gradient in biomarker responses in small mammals collected at different distances from a highway

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Timo Hamers[1]
Lidwien AM Smit[1]
Albertus TC Bosveld[2]
Johannes HJ van den Berg[1]
Jan H Koeman[1]
Frederik-Jan van Schooten[3]
Albertinka J. Murk[1]

Small mammal species studied in Chapter 6: predominantly herbivorous bank vole *Clethrionomys glareolus* (Dutch: rosse woelmuis [left]) and strictly carnivorous common shrew *Sorex araneus* (Dutch: bosspitsmuis [right]).

[1]: Wageningen University - Toxicology Group; [2]: Wageningen UR - Alterra Green World Research; [3]: Maastricht University – Department of Health Risk Analysis and Toxicology

Abstract

This study describes biomarker effects in small mammals exposed to traffic emissions. Animals were collected at 10-50 m (site 1), 150-200 m (site 2) and 5 km (site 3) from a very busy highway (A2). To distinguish between routes of exposure, strictly carnivorous common shrews (*Sorex araneus*) and predominantly herbivorous bank voles (*Clethrionomys glareolus*) were collected. As a measure of exposure to polycyclic aromatic hydrocarbons (PAHs), aromatic DNA adduct levels were determined by ^{32}P-postlabeling techniques in tissue from heart, lung, and liver. Lead (Pb), cadmium (Cd), and copper (Cu) levels were analyzed in kidney as a measure of exposure to heavy metals. EROD and PROD activity and retinoid levels were determined in liver as effect biomarkers for exposure to PAHs and polyhalogenated aromatic hydrocarbons (PHAHs). Relatively high Cd levels in *S. araneus* and in particular elevated DNA adduct levels in *C. glareolus* indicated that small mammals at site 3 were exposed to more compounds than at sites 1 and 2 (3≥1>2). The latter effect is probably due to an incidental and actual input of airborne pollutants that is deposited on plant surfaces. By consumption of above ground vegetation, voles are chronically exposed to this pollution. Relatively high background input of PAHs probably hinders that the traffic-related gradient of airborne PAH concentrations found in an earlier study is reflected in DNA adduct levels in small mammals in the present study. Moreover, historical biomarkers for exposure to traffic emissions, such as increased kidney Pb levels, increased hepatic EROD activity, and disturbed hepatic vitamin A homeostasis are no longer applicable to indicate differences in exposure. This is a result of the ban on addition of Pb and chlorinated scavengers to gasoline and of cleaner combustion techniques, which were enforced by law over the past decade. Finally, it is advisable to use only juvenile small mammals for *in situ* monitoring of diffuse pollution because DNA adduct levels increased with age.

Introduction

In The Netherlands, the emission of volatile organic compounds (excluding CH_4) to the air compartment has been estimated to be 301 kton in 1998, including 719 tons of polycyclic aromatic hydrocarbons (CCDM, 2000). These emissions originate mainly from fuel combustion by traffic, industry and energy plants. Air pollution may be dispersed over long distances by atmospheric transport. It is known that pockets of polluted air also appear in areas that are not in the immediate vicinity of sources of pollution. For example, Van Houdt

et al. (1987) demonstrated that the mutagenicity of airborne particulate matter (APM) sampled at non-industrialized locations in The Netherlands originated from industrial areas more than 100 km away. Besides, mutagenicity depended very much on wind directions during sampling. These results indicate that in a densely populated, well-infrastructured area, differences in air pollution may be small between established exposed sites and expected remote clean areas.

To compare differences in exposure and effect between relatively exposed and remote areas, biomarkers were studied in small mammals living at three different distances from a highway. Biomarkers were selected based on their known responses to the following compound groups, which are known to be emitted by road traffic, currently or in the past.

1. Polycyclic Aromatic Hydrocarbons (PAHs) are emitted as a result of incomplete fuel combustion. Formation of bulky aromatic DNA adducts was measured as a biomarker for PAH exposure (*e.g.* Brookes and Lawley, 1964). Based on the assumption that different routes of exposure contribute differently to DNA damage in different target organs, DNA adducts were measured in tissues from heart, lung, and liver.
2. Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs), were expected as side products of fuel combustion in combination with chlorine-containing lead-scavenging additives, which were added to leaded gasoline to prevent coating of the engine and exhaust system with lead (Marklund *et al.*, 1987; Geueke *et al.*, 1999). Ah-receptor mediated induction of hepatic CYP1A activity (measured as ethoxyresorufin O-deethylase [EROD] activity) was analyzed as a biomarker for exposure to PAHs (Owens, 1977), PCDD/PCDFs and planar PCBs (Safe, 1992). Induction of CYP2B activity (measured as pentoxyresorufin O-deethylase [PROD] activity) was measured as a biomarker for exposure to non-planar PCBs (Safe, 1994). Disturbances in hepatic vitamin A homeostasis were measured as a biomarker for PHAHs (*e.g.* Zile, 1992; Palace *et al.*, 2001; Murk *et al.*, 1998). Hepatic retinol (RE) and retinyl-palmitate (RP) levels were measured, being the active and the storage form, respectively, of vitamin A.
3. Kidney levels of lead were measured as biomarker for exposure to lead emitted during combustion of leaded gasoline (Ratcliff, 1981; Chmiel and Harrison, 1981). Besides, kidney levels of cadmium and copper were measured as a general indication of pollution of the sampling sites.

The goal of the present study was to examine whether exposure to traffic emissions leads to biomarker responses in small mammals and to what extent these responses decrease with increasing distances from a highway. Animals were collected at three different sites located 10-50 m (site 1), 150-200 m (site 2), and 5 km (site 3) from highway A2 in The Netherlands. Previous studies on extracts of APM collected next to sites 1 and 3 have confirmed that concentrations of APM and related genotoxic and Ah-receptor-activating potencies were consistently lower at site 3 than at site 1 (Chapter 2), although differences between sites were small (within one order of magnitude). The toxic potency of APM collected at both sites was attributed partly to biodegradable Ah-receptor activating compounds, such as PAHs.

To distinguish between main routes of exposure, strictly carnivorous common shrews (*Sorex araneus*) and predominantly herbivorous bank voles (*Clethrionomys glareolus*) were studied. These species were selected because they represent different trophic levels, with *S. araneus* being exposed mainly via the food chain and *C. glareolus* via large plant surfaces containing deposited particles. Both species have a high turnover, are locally bound to the sampling site (especially the territorial shrew), and were abundant at the selected sampling sites. Beforehand it was expected that increased exposure to compounds belonging to traffic emissions would lead to an increase in DNA adduct levels, EROD and PROD activities, and heavy metal contents and to a decrease in RP levels.

Material and Methods

Sampling sites

Animals were collected at three sites located east of Highway A2 near Everdingen, The Netherlands. Sites 1 and 2 are located 10-50 and 150-200 m from the highway in Autena, a nature conservation area managed by the Zuidhollands Landschap Foundation (Rotterdam). Site 3 is located at 5 km from the highway in De Regulieren, another natural conservation area managed by the Gelderland Trust for Natural Beauty (Arnhem). At all three sites, animals were trapped in bushy vegetation characterized by tall weeds and willow brushwood. The toxic potency of APM collected on sites 1 and 3 during and prior to the small mammal sampling has been studied in another paper (see Discussion). In that particular paper (Chapter 2), sites 1 and 3 were named sites HIGHWAY and BACKGROUND, respectively, for reasons of convenience.

Animals

Small mammals were trapped during three consecutive nights in June using 120 Longworth Lifetraps (Ebingdon on Thames, UK). Traps were filled with hay to comfort the animals and baited with a mixture of peanuts, pieces of carrots, and fried minced beef heart. After one night of prebaiting, traps were set around sunset, checked two times during the night, and unset at sunrise. After each round of checking traps, caught animals were identified in the field and transported to the laboratory for immediate dissection. Animals were killed under ether anesthetization by a heart punctation, and maturity was determined during dissection based on an expert judgment of thymus atrophy and development of the sex organs. Liver, lungs, heart, and kidneys were collected and frozen immediately in liquid N_2. All organs were stored at –20°C, except for the liver, which was stored at –80°C.

DNA isolation

A small piece of heart, lung or liver was cut into smaller pieces and lysed with 400 µl SET/SDS (100 mM NaCl, 20 mM EDTA, 50 mM Tris, 0.5% SDS, pH=8.0) at 37°C for 3 h. The resulting suspensions were treated with 50 µl RNase mixture (0.1 mg/ml RNase A and 1000 U/ml RNase T1 in SET/SDS) for 1 hour at 37°C, and only liver suspensions were subsequently incubated for another hour with 50 µl of α-amylase (10 mg/ml in SET/SDS) at 37°C. All suspensions were further treated with 50 µl proteinase K (10 mg/ml) and incubated overnight at 37°C. DNA was isolated by subsequent extractions with 500 µl phenol, phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) and then precipitated with two volumes of 100% cold ethanol and 1/30 volume of 3 M sodium acetate (pH=5.3). Precipitated DNA was rinsed with 70% ethanol and dissolved in 2 mM Tris (pH=7.4). Quality and quantity of the DNA were determined spectrophotometrically ($A_{260/230}$~2.3, $A_{260/280}$ ~1.8) and its concentration was adjusted to 2 mg/ml.

^{32}P postlabeling assay

The postlabeling assay was performed based on the method originally described by Reddy and Randerath (1986): 5 µl of DNA (2 mg/ml) was digested into deoxyribonucleoside 3'-monophosphates by incubation for 3.5 h at 37°C with 5 µl digestion mixture (calf spleen phosphodiesterase [2 µg/µl], micrococcal endonuclease [0.25 U/µl], sodiumsuccinate [100 mM], $CaCl_2$ [5 mM]). Half of the digest was treated with 5 µl nuclease P1 (2.5 µg/µl in sodium acetate [2.5 M] and $ZnCl_2$ [0.3mM]) for 40 min. Nuclease P1 treatment was stopped

with 2.5 µl Tris (0.5 M) and the 3' phosphorylated nucleotides are subsequently labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase (10 U/µl in kinase buffer). Radiolabeled adducted nucleotide biphosphates were separated by two-dimensional chromatography of 14 µl reaction mixture on polyethylene-imine (PEI)-cellulose sheets (Macherey Nagel, Düren-Germany), using the following solvent systems: D1: 1 M NaH_2PO_4, pH=6.5; D2: 8.5 M urea, 5.3 M lithium formate, pH=3.5; D3: 1.2 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH=8.0; D4: 1.7 M NaH_2PO_4, pH=6.0. To ensure the efficiency of the nuclease P1 treatment and ATP excess, the remaining 1.5 µl of the digest was one-dimensionally chromatographed on PEI-cellulose sheet (Merck, Darmstadt, Germany) using a solvent system of 0.12 M NaH_2PO_4, pH=6.8. For quantification purposes, two standards of benzo[a]pyrene-diol-epoxide (BPDE)-DNA standards (1 adduct per 10^7 or 10^8nucleotides) were run in parallel in all chromatographies. Quantification was performed using phosphorimaging technology (Molecular Dynamics™, Sunnyvale CA). Quantitatively, half of the detection limit for the diagonal radioactive zone (=0.5 * 1 adduct per 10^8 nucleotides) was considered as the determined level of adducts for samples that showed neither a diagonal radioactive zone (DRZ) nor an adduct spot in their adduct maps. Nucleotide quantification was done by labeling the remaining half of the digested DNA with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase and by subsequent separation of the nucleotides by one-dimensional chromatography on PEI-cellulose sheet using the solvent system of 0.12 M Na_2PO_4 (pH=6.8).

Preparation of liver homogenates and microsomes

Livers were homogenized in ice-cold 1:2 (w/v) Tris-buffer (10 mM Tris-HCl; 0.25 M sucrose; 1 mM dithiothreiotol (DTT); pH=7.4) using a laboratory homogenizer (Potter tube). Two aliquots of 50 µl homogenate were frozen at –20°C for retinoid measurements. The rest of the homogenate was centrifuged for 30 min (12000g; 4°C; Kotron Centricon T-1055) and after removal of floating fat the supernatant was centrifuged for 75 min (105000g; 4°C). The microsomal pellet was resuspended in ice-cold Tris-buffer (10 mM Tris-HCl; 3 mM EDTA; 1 mM DTT; pH=7.4) and frozen in aliquots of 175 µl at -80°C (3 µl per mg liver weight [*S. araneus*] or 1000 µl irrespective of liver weight [*C. glareolus*]). Protein content of the microsomal fraction was determined by the method of Bradford (1976), which was adapted to use in a 96-well microtiter plate.

Enzyme assays

EROD and PROD activity were measured according to the method described by Burke *et al.* (1977), which was adapted for use in a 96-well microtiter plate. Reaction mixtures contained 0.4 µM ethoxyresorufin (ER) or pentoxyresorufin (PR), 0.1 mM NADPH, 1 mg/ml bovine serum albumine (BSA) and 100 µg/ml microsomal protein in a final volume of 200 µl 0.1 M Tris-HCl buffer (pH=7.8). After preincubation for 2 min (37°C), the reaction was started by the addition of 50 µl 0.4 mM NADPH in Tris (pH=7.8). Background fluorescence was measured immediately (t=0) and the plate was further incubated on a plate-shaker. The formation of the product resorufin (RR) was followed fluorimetrically (λ_{ex}=530 nm, λ_{em}=590 nm) on a Cytofluor 2350 plate-reader (Millipore, NL) by consecutive measurements at t=5, t=10 and t=15 min. Fluorescence was interpolated in a calibration curve of the 0-150 nM dilution series of RR (in 0.1 M Tris-HCl buffer (pH=7.8) containing 1 mg/ml BSA), which was measured simultaneously in the microtiter plate. Incubations were carried out in duplicate, and results are expressed as pmol RR produced per mg protein per min.

Hepatic Vitamin A analysis

Retinol and retinyl-palmitate levels were measured in liver homogenates according to Brouwer *et al.* (1989) with some modifications. Liver homogenates were four times (*S. araneus*) or three times (*C. glareolus*) diluted in Tris-buffer (10 mM Tris-HCl; 3mM EDTA; 1mM DTT; pH=7.4). Duplicate aliquots (50 µl) of diluted homogenate were extracted in a 1:2:1 volume ratio with diisopropylether (DIPE) and methanol containing the internal standard retinylacetate (1 µg/ml) and 0.1% butyl-hydroxytoluene (BHT) as an antioxidant. Samples were vortexed for 30 s and kept overnight at –20°C, then vortexed again and centrifuged in an Eppendorf centrifuge (5000 rpm, 5 min). The DIPE-phase was collected and filtered over a 0.45 µm filter (Millipore, NL), evaporated under N_2 and redissolved in 100 µl ethylacetate/methanol (1:3) with 0.1% BHT. Extraction efficiencies were routinely above 80%. Aliquots (20µl) of dissolved extracts were analyzed with high performance liquid chromatography (HPLC) using a C18 analytical column (Pecosphere 3 µm particle size, 3.3 cm length, 4.6 mm internal diameter; Perkin Elmer) and a wavelength of 326 nm for detection of retinoids. Retinoids were analyzed by 86% methanol and 14% water for 1.5 min, followed by a gradient to 100% methanol for 2.5 min, and subsequent elution of the retinyl esters for 12 min. The column was then equilibrated again at starting conditions for 6 min.

Heavy metal analysis

Heavy metal analysis was performed as described earlier by Schilderman *et al.* (1997). Kidney samples were dried overnight at 95°C and subsequently ground powder using an agate mortar. Each sample was treated with 5 ml of 1:1 mixture of 65% HNO_3 (Merck, p.a.) and water and subsequently boiled for 2 h in a water bath (100°C). After filtration (Schleicher & Schuell 589 black band filter), the filtrate was evaporated to moist residue (2-3 ml) and subsequently diluted with 0.1% HNO_3. Concentrations of Cd, Pb, and Cu were measured by graphite furnace AAS with Zeeman background correction at 228.8, 283.3 and 327.4 nm, respectively. Ashing and atomization temperatures were 400°C and 1800°C for Cd, 500°C and 2300°C for Pb, and 800°C and 2300°C for Cu. Cd-, Pb- and Cu-nitrate in 0.5 M HNO_3 were used for calibration. Paladium nitrate was used in all instances as a modifier. All glassware was rinsed with 1% HNO_3 to avoid contamination.

Data analysis

Effects of sampling site were estimated by ANOVA for normally distributed (Kolmogornov-Smirnov test) ln-transformed results with equal variances (Levene's test). To correct for (possible) side effects of age differences on biomarker results within the heterogeneous sampling populations (Table 1), bodyweight was introduced as a covariate into all ANOVA analyses. Site differences were determined by pairwise comparisons of estimated marginal means, adjusted for multiple comparisons by Bonferroni correction. Nonparametric tests were used for heteroscedastic or not-normally distributed results: Mann-Whitney (k=2) and Kruskal-Wallis (k>2) test were applied for independent samples. Differences within animals (*e.g.* between DNA adduct levels in heart and lung) were tested using Wilcoxon signed rank (k=2) and Friedman's (k>2) test for related samples. In case of nonparametric tests with k>2, Tukey-type multiple comparisons were performed using Nemenyi's test (Zar, 1996). In Figures and Tables, data are presented as retransformed averages with 95% confidence intervals. Correlations mentioned in the text refer to ln-transformed data, except for bodyweights. Actual p-values have been mentioned in the results section. All statistical calculations were performed using SPSS Software Package (Release 10.1, 2001).

Results

Sampling population

Out of a total of 96 small mammals trapped, 33 individuals were identified as common shrew *S. araneus* and 54 as bank vole *C. glareolus*. Additional catches concerned the field vole *Microtus arvalis* (5) and the common vole *M. agrestis* (4). At the time of sampling (June), the two most abundant species clearly demonstrated a different population structure (Table 1). For *S. araneus*, most individuals (73%) were classified as juvenile, with none of the female animals being pregnant. For *C. glareolus*, most individuals (75%) were classified as adult, with 36% of all female animals being pregnant. At site 3, no adult *S. araneus* were caught, and numbers of *C. glareolus* were small.

Due to logistic problems, DNA adducts were measured in lung from only 27 individuals (*S. araneus* and *C. glareolus*) and in liver from 32 *C. glareolus* individuals. Liver DNA adducts were not measured in *S. araneus*, because all hepatic tissue was required for EROD, PROD, and vitamin A analyses. All other biomarkers were measured in all 33 *S. araneus* and 54 *C. glareolus* individuals caught.

Table 1: Number of animals collected at different sites[a].

Location	*Sorex araneus*				*Clethrionomys glareolus*			
	male		female		male		female	
	adult	juvenile	adult	juvenile	adult	juvenile	adult	juvenile
1	1	1	3	3	12	5	7 (3)	1
2	1	6	4	6	9	1	10 (4)	5
3	-	1	-	7	1	1	2 (2)	-

[a]: Numbers in parentheses indicate numbers of pregnant females.

DNA adducts

For all three tissues (heart, lung, liver) from both species analyzed, autoradiographic profiles of the chromatograms showed Diagonal Radioactive Zones (DRZs), which are typical for exposure to complex mixtures of PAHs. Intense and stretched DRZs were often found in heart tissue, whereas compound specific spots were sometimes noted especially in lung tissue.

DNA adduct levels in *S. araneus* ranged from below detection limit (n=1 in heart) to 10.1 and 6.6 adducts per 10^8 nucleotides in heart and lung tissue, respectively. In heart and lung tissue, DNA adduct levels did not differ significantly among sampling sites, although average adduct levels clearly declined with increasing distance from the highway (Table 2). However, this effect could not be attributed to different sampling sites but was completely explained by

age differences among sampling populations. No adult individuals were collected at site 3 (Table 1), and bodyweight appeared to be a significant age-indicative covariate for both heart and lung adducts. DNA adduct levels significantly correlated with bodyweight in heart ($r=0.67$; $p<0.001$) and in lung ($r=0.50$; $p=0.008$) (Figure 1). Within *S. araneus* individuals, levels of DNA adducts in heart tissue were greater than in lung tissue ($p=0.034$), with a significant correlation ($p=0.011$) of $r=0.48$ between both tissues. In both tissues, DNA adduct levels were independent from sex.

DNA adduct levels in *C. glareolus* ranged from below detection limit (n=2 in heart) to 7.1, 7.5 and 6.8 adducts per 10^8 nucleotides in heart, lung, and liver tissue, respectively. Site effects on DNA adduct levels were only found in lung tissue, with slightly greater levels ($p=0.092$) in mice from site 3 than from sites 1 and 2 (Table 2). In fact, in all three tissues, average DNA adduct levels declined in the order of site $3 \geq 1 > 2$, with most prominent differences among the sampling sites found for the subgroup of adult female *C. glareolus* (Table 2; $p=0.021$, $p=0.010$ and $p=0.037$, respectively). DNA adduct levels significantly correlated with bodyweight in heart ($r=0.44$; $p=0.001$) and liver ($r=0.42$; $p=0.017$), but not in lung. DNA adduct levels in lung tissue were significantly greater than in heart and liver tissue ($p=0.002$; Table 2). Adduct levels in heart and lung correlated significantly ($r=0.54$; $p=0.004$) in the predominantly adult subpopulation for which both tissues were analyzed (Figure 2). In all three tissues tested, DNA adduct levels were independent from sex or pregnancy.

Hepatic enzyme assays

For both species, EROD activity in microsomal liver fractions was on average 12.5 and 6.5 times greater than PROD activity (Table 2), with good correlations ($p<0.001$) between both enzymatic activities ($r=0.75$ for *S. araneus* and $r=0.65$ for *C. glareolus*). EROD and PROD activity were significantly greater in the herbivorous voles than in the carnivorous shrews ($p<0.001$) but no site effects were observed (Table 2).

For *S. araneus*, EROD and PROD activity weakly correlated with bodyweight ($r=-0.37$ ($p=0.032$) and $r=-0.34$ ($p=0.054$), respectively). Irrespective of bodyweight, EROD activities (not PROD) were slightly greater in male than in female *S. araneus* ($p=0.088$). In *C. glareolus* liver, neither EROD nor PROD activities depended on bodyweight, sex, or pregnancy.

Table 2: Average biomarker responses and heavy metal concentrations on sites 1, 2 and 3 (with 95% confidence intervals in parentheses), back-transformed from the originally ln-transformed data[a].

	Sorex araneus (all)			*Sorex araneus* (juvenile ♀♀)			*C. glareolus* (all)			*C. glareolus* (adult ♀♀)		
	1	2	3	1	2	3	1	2	3	1	2	3[b]
	N=8	*N*=17	*N*=8	*N*=3	*N*=6	*N*=7	*N*=25	*N*=25	N=4	*N*=7	*N*=10	*N*=2
DNA adducts (numbers per 10^8 nucleotides)												
Heart	3.4 (1.9-6.2)	2.6 (1.9-3.7)	2.1 (1.3-3.6)	2.2 (0.8-5.6)	1.7 (1.1-2.4)	2.1 (1.1-4.0)	2.5 (2.0-3.1)	2.1 (1.6-2.8)	2.6 (0.8-8.7)	**3.4 AB** (2.1-5.5)	**2.4 B** (1.8-3.2)	**4.9 A** (0.5-49)
Lung	2.6 (1.7-4.1) *n*=8	2.7 (1.8-4.0) *n*=11	1.4 (1.0-2.1) *n*=8	1.7 (0.4-7.4) *n*=3	1.9 (0.9-3.8) *n*=7	1.5 (0.9-2.3) *n*=7	**3.3 ab** (2.6-4.1) *n*=13	**2.9b** (2.1-3.8) *n*=10	**4.8 a** (2.0-11.6) *n*=4	**4.0 B** (1.8-9.1) *n*=7	**2.8 B** (2.2-3.7) *n*=8	**7.4 A** (5.5-9.8) *n*=2
Liver	-	-	-	-	-	-	2.2 (1.5-3.1) *n*=14	1.7 (1.3-2.3) *n*=14	2.7 (1.2-6.1) *n*=4	**3.2 A** (1.2-8.6) *n*=4	**1.3 B** (0.8-2.2) *n*=8	**4.2 A** (4.0-4.5) *n*=2
Hepatic cytochrome P450 activity (pmol resorufine produced per mg protein per minute)												
EROD	30 (11-83)	44 (34-56)	40 (32-52)	70 (7.6-654)	44 (23-83)	39 (30-52)	103 (81-129)	94 (83-108)	104 (30-359)	104 (70-156)	102 (74-140)	173 (0.13-228937)
PROD	5.7 (4.5-7.1)	6.4 (6.0-7.0)	5.6 (4.9-6.5)	6.4 (3.0-13.6)	6.9 (5.8-8.2)	5.6 (4.7-6.5)	7.9 (7.1-8.7)	7.6 (7.1-8.3)	8.1 (4.7-13.9)	8.1 (6.8-9.8)	7.7 (6.7-9.0)	10.0 (0.38-264)
Kidney levels of heavy metals (mg/kg dw)												
Cd	**8.6 AB** (2.4-31)	**4.6 B** (2.5-8.4)	**8.4 A** (6.1-11.6)	**2.0 B** (0.1-47)	**2.3 B** (1.5-3.7)	**8.6 A** (5.9-13)	1.9 (1.2-3.1)	1.9 (1.2-3.0)	1.4 (0.04-56)	3.6 (1.4-9.6)	3.2 (1.6-6.3)	7.3 (3.0-18)
Pb	2.6 (0.5-13)	1.1 (0.5-2.5)	0.3 (0.1-1.0)	1.7 (0.01-322)	1.0 (0.1-9.1)	0.3 (0.1-0.6)	1.9 (1.2-3.1)	1.1 (0.6-2.0)	2.1 (0.1-40)	3.1 (1.2-8.2)	1.2 (0.5-3.3)	3.3 (2.1-5.0)
Cu	33 (24-46)	31 (26-36)	32 (26-40)	36 (7-173)	28 (22-36)	32 (25-42)	16 (13-18)	16 (15-18)	15 (10-19)	18 (14-23)	16 (14-17)	15 (0.9-256)
Hepatic retinoid levels (ng/mg ww)												
RE	**1.9 AB** (1.1-3.2)	**1.0 B** (0.6-1.6)	**3.4 A** (2.2-5.4)	**1.6 AB** (0.5-5.0)	**0.7 B** (0.2-2.4)	**3.2 A** (2.0-5.1)	**1.7 B** (1.2-2.3)	**2.4 A** (1.9-3.1)	**1.8 AB** (1.0-3.2)	2.2 (1.2-4.2)	3.3 (2.3-4.8)	1.5 (0.4-5.4)
RP	8.4 (4.6-15.4)	3.9 (5.1-9.4)	10.0 (5.9-17.0)	7.5 (1.1-50)	6.2 (4.1-9.4)	10.2 (5.4-19.2)	**99 ab** (67-145)	**106 b** (74-150)	**52 a** (12-221)	151 (64-357)	165 (103-265)	83 (1.0-7157)

[a]: For each species, results are presented for all animals collected and for the most predominant subgroup per species, *i.e.* juvenile *S. araneus* females and adult *C. glareolus* females. Significant site effects are printed **in bold**, with differences between the sampling sites being indicated with different CAPITAL ($p<0.05$) or small ($p<0.10$) characters. Unless stated differently, sample sizes (*n*) are equal to the number of animals collected (*N*), given at the top of each column. DNA-adducts were not determined in *S. araneus* livers because all liver tissue was required for EROD, PROD and Vitamin A analyses.

[b]: Extremely large confidence intervals are due to the fact that only 2 adult female *C. glareolus* were collected at site 3.

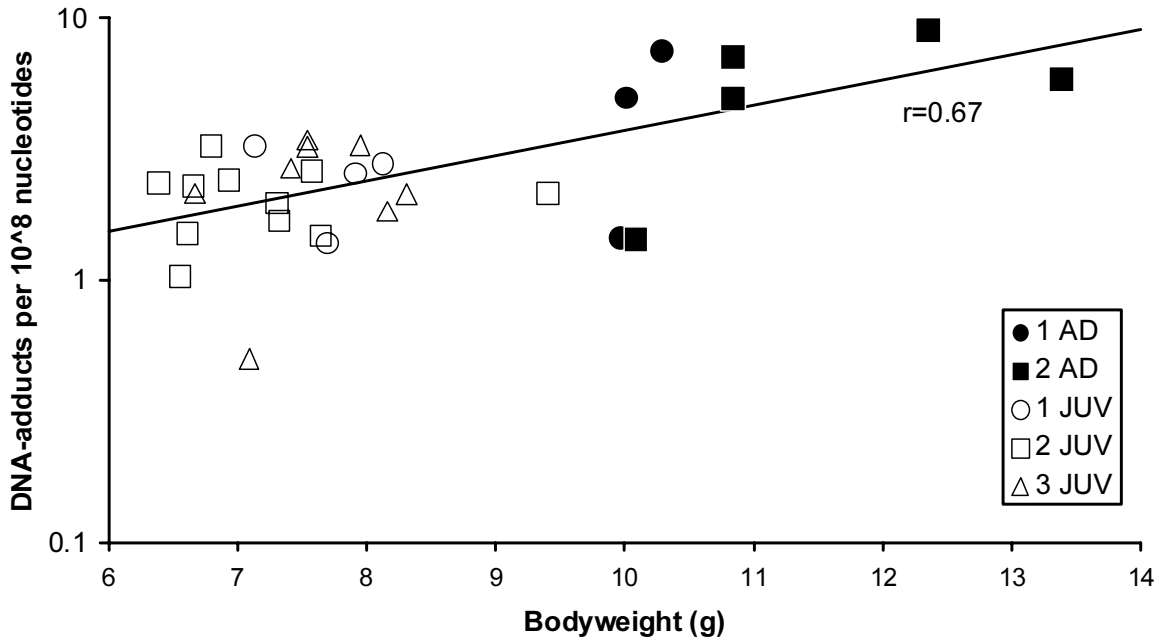

Figure 1: Positive correlation (r=0.67; p<0.001) between heart DNA adduct levels and bodyweight in *S. araneus*. A similar significant correlation (r=0.44; p=0.001) was found for *C. glareolus*. Solid and open markers refer to adult (AD) and juvenile (JUV) animals. Circles, squares and triangles refer to animals from sampling site 1, 2, or 3, respectively.

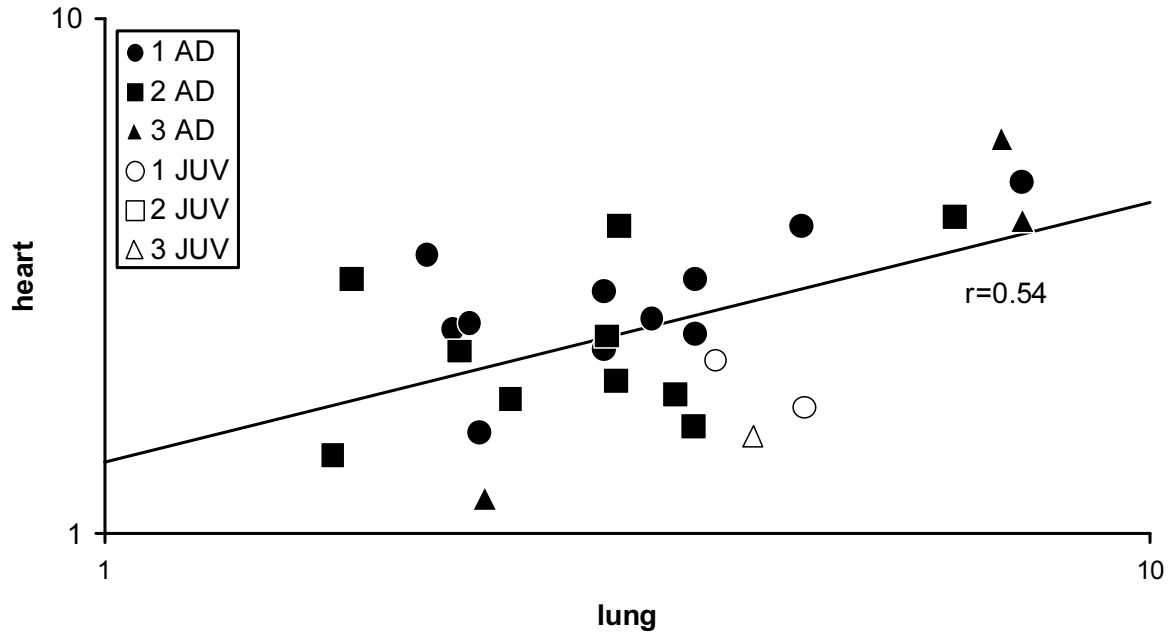

Figure 2: Positive correlation (r=0.54; p=0.004) between DNA adduct levels (number per 10^8 nucleotides) in heart and lung in *C. glareolus*. A similar significant correlation (r=0.48; p=0.011) was found for *S. araneus*.

Heavy metal contents

In kidney tissue from *S. araneus*, heavy metal concentrations significantly correlated with bodyweight for Cd ($r=0.65$; $p<0.001$) and Pb ($r=0.42$, $p=0.016$), but not for Cu. Site effects were only found for Cd, with greatest levels in juvenile shrews from site 3 ($p=0.008$; Table 2). Average Pb levels in *S. araneus* seem to decline with increasing distance from the highway, but, similar to the heart adduct levels, this difference was completely explained by the fact that no adult shrews were collected at site 3. No site effects were found on Cu levels in kidneys from *S. araneus*.

In *C. glareolus*, heavy metal levels did not differ among the sampling sites (Table 2). Cd levels significantly correlated with bodyweight ($r=0.61$; $p<0.001$), but Pb and Cu levels did not. Finally, Cd and Cu levels were significantly greater in *S. araneus* than in *C. glareolus* ($p<0.001$), whereas Pb levels were similar in both species (Table 2). In both species, levels of heavy metals were independent of sex or pregnancy.

Hepatic Vitamin A analysis

Average hepatic RP levels in *C. glareolus* (97 ng/mg) were significantly greater ($p<0.001$) than in *S. araneus* (7.9 ng/mg), but average RE levels did not differ between both species (2.0 and 1.5 ng/mg, respectively) (Table 2). For *S. araneus*, RE levels were significantly greater at site 3 than at site 2 ($p=0.004$), but RP levels did not differ among sites (Table 2). A weakly inverse correlation was found between RP levels and EROD activity ($r=-0.37$; $p=0.083$) in female *S. araneus* (not in male). No relationship was found between RE levels and EROD activity. RE and RP levels were independent of age, but RP levels in juveniles appeared to be significantly lower in male than in female *S. araneus*.

In *C. glareolus*, hepatic retinoid levels significantly correlated with bodyweight: Correlation coefficients of $r=0.55$ ($p<0.001$) and $r=0.31$ ($p=0.025$) were calculated for RP and RE levels, respectively. Retinoid levels also differed among sampling sites (Table 2), with lower RE levels at sites 1 than at site 2 ($p=0.044$) and lower RP levels at site 3 than at sites 1 and 2 ($p=0.064$). As in female *S. araneus*, a significant inverse correlation was found between hepatic RP levels and EROD activity ($r=-0.51$; $p=0.025$) in the subgroup of adult female *C. glareolus*. Hepatic RE and RP levels in *C. glareolus* were independent of sex or pregnancy.

Summary

An overview of the significant biomarker differences is presented in Table 3. For each species, results have been presented for all individuals tested as well as for the largest homogeneous subsets of individuals, *i.e.* juvenile female *S. araneus* and adult female *C. glareolus* (Table 1).

Table 3: Summary of all significantly different biomarker responses or heavy metal concentrations from Table 2[a].

Species	Parameter	3>1>2[b]	*n* (3-1-2)[c]
S. araneus all	kidney Cd-levels	A-AB-B	8-17-8
S. araneus juvenile ♀♀	kidney Cd-levels	A-B-B	7-3-6
C. glareolus all	lung DNA-adduct levels	a-ab-b	4-13-10
	hepatic retinyl palmitate levels	A-AB-B	4-25-25
C. glareolus adult ♀♀	heart DNA-adduct levels	A-AB-B	2-7-10
	lung DNA-adduct levels	A-B-B	2-7-8
	liver DNA-adduct levels	A-A-B	2-4-8

[a]: Sites 1, 2, and 3 were located at 10-50 m, 150-200 m, and 5 km east from the highway, respectively. All differences indicate that exposure is higher at site 3 than at site 1 than at site 2 (3>1>2). Differences in hepatic retinol levels were excluded (see Discussion).

[b]: Differences between sites are indicated by different CAPITAL ($p<0.05$) or small ($p<0.10$) characters.

[c]: number of animals analyzed from site 3, 1, and 2, respectively.

Discussion

Differences among sites

For all biomarkers selected in this study, measurable responses were found, but differences in response were small among sampling sites (Table 2). As expected, results suggest on average that small mammals are exposed to more traffic emissions at site 1 next to the highway than at site 2 located 150-200 m from the highway (1>2). However, all significant differences in biomarker responses indicated that animals at background location site 3 were exposed to more compounds than sites 1 and 2 (Table 3). This pattern of pollution (3≥1>2) is in contrast with the expected pollution pattern (1>2>3). Although only four *C. glareolus* individuals were collected at site 3, the pollution pattern 3≥1>2 is especially reflected in this species by significantly increased lung DNA adduct levels and by decreased hepatic RP levels (Table 3), which were negatively correlated to EROD activity in adult female mice. It is further supported by non-significant differences in DNA adduct levels in heart and liver, hepatic EROD and PROD activity, and kidney levels of Pb (Table 2).

In contrast to other studies where PHAH exposure differed greatly and resulted in RP and even RE effects (*e.g.* Murk *et al.*, 1998; Palace *et al.*, 2001), no differences in exposure

(measured as EROD activities) were found among the three sites. Therefore it was to be expected that RE levels and EROD activity would not correlate. However, the more univocal decrease in hepatic RP levels did correlate inversely with EROD activity. Therefore RP effects were included in Table 3, but RE effects were not.

Apparently, site 3 is exposed to some kind of background pollution, which (1) is not related to traffic, (2) is especially available for herbivorous voles, and (3) is composed of a complex mixture of compounds, including PAHs, planar PHAH-like pollutants (given the differences in DNA adduct and RP levels, respectively), and heavy metals. The origin of this extra input at site 3 is probably airborne, because there is no history of on site pollution in this nature conservation area. As the response levels are greater at site 3 than at site 2, it is likely that the input originates from a local source.

Main route of exposure

To distinguish the most critical route of *in situ* animal exposure to PAHs, levels of aromatic DNA adducts have been measured in different tissues. Heart, lung, and liver tissue are exposed to DNA-damaging agents present in the blood circulation, but lung and liver tissue are additionally exposed to compounds present in inhaled air and in digested food, respectively. For both species tested, significant correlations were found between heart and lung adduct levels, with most pronounced differences among sites in lung tissue. Especially in (adult) *C. glareolus*, DNA adduct levels were greater in lung than in heart and liver tissue, with lung adduct levels clearly demonstrating the 3≥1>2 pollution gradient between sites.

Formation of DNA adducts in lung tissues may be caused by direct intratracheal exposure of lung tissue to PAHs (Godschalk *et al.*, 2000) but also by dermal (Savela *et al.*, 1995; Randerath *et al.*, 1999) and oral exposure (*e.g.* Weyand *et al.*, 1991 and 1994; Bordelon *et al.*, 2000). Inhalation is not likely to be the most important route of PAH exposure for *C. glareolus* at site 3 because BaP-equivalent concentrations of APM are known to be consistently lower than at site 1 (Chapter 2). Dermal contact to deposited PAHs is also reasoned to be not the most important route of exposure; in an earlier laboratory study no lung DNA adduct were found with rats exposed to litters of notoriously PAH polluted soils (Fouchécourt *et al.*; 1999). The fact that pollution gradient 3≥1>2 is only reflected in lung from *C. glareolus* and not from *S. araneus* further excludes inhalation and dermal contact as the main route of exposure, because both species inhale the same air and dwell in the same vegetation and litter layer. Thus, oral uptake is the only alternative important route of

exposure for *C. glareolus* at site 3. This hypothesis is supported by a relative high correlation between lung and liver adducts at site 3 (r=0.88) compared to sites 1 and 2 (r=-0.32 and r=0.14, respectively). At site 3, liver DNA adducts also correlated relatively well with hepatic EROD-activities (r=0.74) compared to sites 1 and 2 (r=0.09 and r=-0.06). Such a correlation was found earlier (*e.g.* Fouchécourt *et al.*, 1999) and was to be expected, as many PAHs induce CYP450 and need to be metabolized before they can bind covalently to DNA. Unfortunately, too few individuals of C. *glareolus* were collected at site 3 (n=4) to prove the significance of these correlations.

If oral uptake is indeed the most important route of exposure at site 3, this also explains why pollution gradient 3≥1>2 found in *C. glareolus* is not reflected in *S. araneus*. Herbivorous *C. glareolus* feed on plants, whereas carnivorous *S. araneus* mainly prey on earthworms, soil-dwelling insects, snails, and slugs. Apparently, bioaccumulation of PAHs in prey species and their subsequent transfer through the short food chain to the carnivorous shrews is less important than the consumption of relatively large plant surfaces with deposited PAHs, leading to a chronic oral exposure of herbivorous small mammals. The difference between both species may therefore indicate that exposure to PAHs at site 3 is not through the soil but through consumption of above-ground vegetation. This confirms the hypothesis that the input of compounds at site 3 is an incidental, actual airborne input, rather than a historical pollution.

Traffic emissions and DNA adducts

DNA adduct levels did not decrease with increasing distance from the highway. This was in contrast to the expectation based on toxic potencies of airborne particulate matter (APM) collected next to sites 1 and 3 during and prior to the small mammal trapping period (Chapter 2). APM extracts were measured in the DR-CALUX assay (Dioxin-Responsive Chemical-Activated Luciferase gene Expression), a reporter gene assay based on a hepatoma cell line of rat (H4IIE) stably transfected with a vector containing a luciferase gene under transcriptional control of the Dioxin Responsive Elements (DRE) (Aarts *et al.*, 1995). Exposure of DR-CALUX cells to Ah receptor agonists induces luciferase production, which is quantified by measuring light production after addition of the substrate luciferin. By adopting different exposure times in the DR-CALUX protocol, Ah-receptor activation by easily biodegradable compounds in the APM extracts (*e.g.* PAHs) can be distinguished from relatively persistent compounds (*e.g.* PHAHs). Based on calibration curves measured for the reference PAH

benzo[a]pyrene (BaP) and the reference PHAH 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), DR-CALUX responses to APM samples were recalculated into BaP or TCDD equivalent concentrations (pmol or fmol per m^3 of air sampled) (Chapter 2).

On corresponding sampling days, DR-CALUX responses were consistently greater for APM extracts from site 1 than from site 3, with toxic potencies being equivalent to airborne concentrations of 0.91-14.8 and 0.86-4.9 ng BaP/m^3, respectively. DR-CALUX responses were completely attributed to biodegradable compounds, such as nitro-PAHs, because responses were below detection limit when testing under conditions specific for persistent Ah-receptor agonists (Chapter 2). However, differences among sites 1 and 3 in airborne PAH concentrations are not reflected in DNA adduct levels in the present study. Similarly, Schilderman *et al.* (1997) also found no relationship between DNA adduct levels in lung, liver, or kidney from urban pigeons and airborne concentrations of adduct yielding PAHs at sampling sites with high, medium, and low traffic density (2.9, 1.3, and ≤1.1 ng PAH/m^3, respectively). Schilderman *et al.* (1997) concluded that DNA adduct levels in pigeons do not seem to be a relevant biomarker for inhalatory exposure to mutagenic and carcinogenic PAHs emitted by traffic. The present study shows that this conclusion also holds for small mammals. Probably differences in airborne DNA binding PAHs at different distances from the highway are too small to be reflected in DNA adduct levels.

DNA adducts in feral animals

As far as we know, this is the first study applying ^{32}P-postlabeling techniques to determine DNA adduct levels *in situ* in terrestrial small mammals. Apparently, ^{32}P-postlabeling techniques are suitable for measuring DNA adducts in feral small mammals, because in only three of all tested samples adduct levels were below the detection limit. Usually, these techniques are used in laboratory studies where laboratory animals are exposed to well-known mutagens under controlled conditions. Routinely, mice and rats are used as test animals, but some studies have been performed on toad larvae (Jones and Parry, 1992) and mussels (Kurelec *et al.*, 1988; Venier and Canova, 1996; Canova *et al.*, 1998), which are more representative species for the freshwater and marine aquatic environment, respectively. So far, studies on DNA adducts for monitoring of environmental exposure to PAHs have mainly focused on humans (*e.g.* Binkova *et al.*, 1995; Godschalk *et al.*, 1998) and fish (*e.g.* Kurelec *et al.*, 1989; Van der Oost *et al.*, 1994; Van Schooten *et al.*, 1995; Ericson *et al.*, 1999). Other species have been studied, but sparsely: DNA adduct levels have been measured

in earthworms for *ex situ* monitoring of polluted soils (Van Schooten *et al.*, 1995; Walsh *et al.*, 1997), and in pigeons (Schilderman *et al.*, 1997) and muskrats (Halbrook *et al.*, 1992) for *in situ* monitoring of exposure to PAHs. Average DNA adduct levels in feral animals exposed to mixtures of PAHs under field conditions range from 0.16 in perch *Perca fluviatilis* (Ericson *et al.*, 1999) to 34.3 adducts per 10^8 nucleotides in eel *Anguilla anguilla* (Van Schooten *et al.*, 1995). The levels found in the present study for *S. araneus* and *C. glareolus* fit well within this range reported for different organisms (Table 2).

Age-dependent DNA adducts

In the present study, levels of DNA adducts significantly correlated with bodyweight in all organs of both species tested (except for lung tissue from *C. glareolus*), suggesting that formation of DNA adducts is (partly) age dependent. Possibly, longer PAH exposures of adult than juvenile animals lead to more accumulation of DNA adducts in adults. Alternatively, in untreated rodents covalent DNA modifications of endogenous origin (called I-compounds) have been described, which increased with age and were tissue-, species-, and sex dependent (Nath *et al.*, 1996). In wildlife Californian sea lions (*Zalophus californicus*) (Reichert *et al.*, 1997) and in various feral aquatic vertebrate and invertebrate animal species (Garg *et al.*, 1992), similar "natural" occurring DNA modifications have been found that were age-dependent or season-dependently associated with reproductive cycles. Such endogenously formed DNA adducts may interfere with ^{32}P-postlabeling detection of low levels of DNA adducts arising from anthropogenic contamination. They may prevent the establishment of a quantitative relationship between DNA adduct levels and contaminated field sites as found for small mammals in the present study and for many fish species and muskrats in earlier studies (Kurelec *et al.*, 1989; Van der Oost *et al.*, 1994; Halbrook *et al.*, 1992). To minimize interference by accumulated DNA damage and age-dependent endogenous DNA adducts, it may be better to use juveniles instead of adults for biomonitoring purposes.

Species differences

Some parameters differed significantly between both species, irrespective of the sampling site (Table 2). On average, kidney concentrations of cadmium were four times greater in *S. araneus* than in *C. glareolus*. Bioaccumulation of heavy metals in insectivores is a well-known phenomenon. Ma *et al.* (1991) found that kidney concentrations of cadmium and lead were two orders and one order of magnitude greater, respectively, in *S. araneus* than in field

vole *Microtus agrestis* irrespective of the degree of pollution at the sampling site (flood plains). In our study, differences between species in Cd levels were smaller but still significant, whereas Pb levels did not differ among species. Differences are possibly smaller because *M. agrestis* is a stricter herbivore than C. *glareolus* or because exposure of *S. araneus* to heavy metals differed between both studies. Kidney levels of Cd in *S. araneus* are comparable to control levels reported by Ma *et al.* (1991), whereas Pb levels are remarkably lower.

Cu levels were also significantly greater in *S. araneus* than in *C. glareolus*. Because Cu is an essential element, these species differences are not necessarily due to differences in diet-related bioaccumulation. They can also be explained by differences in species-specific Cu homeostasis. Kidney Cu levels in our study are in good correspondence with levels reported by Pankakoski *et al.* (1994), who also found greater Cu levels in *S. araneus* than in *C. glareolus* but no differences between animals from a lead smelter area and a control site.

Hepatic EROD activity and RP levels were about one order of magnitude greater in *C. glareolus* than in *S. araneus*. Higher EROD activity in herbivorous than carnivorous species were to be expected, as plant food contains many phyto-aromatic compounds, which need to be metabolized by an active P450 system (Nebert *et al.*, 1989). Differences between species in hepatic RP levels can also be explained by differences in food choice. For herbivorous species, the main source of vitamin A are carotenoids present in plant food, which are further metabolized into RE, which again is stored in the liver as RP. Carnivorous species mainly obtain their vitamin A from RP stores in their prey. Especially consumption of liver from prey leads to extremely high hepatic RP levels in top predators. The fact that prey species from first-order carnivorous *S. araneus* do not have a liver and a consequent hepatic RP store may explain the lower RP levels in liver from carnivorous shrew than from herbivorous bank vole.

Reduced traffic emissions

Compared to earlier available studies on traffic emissions (*e.g.* Chmiel and Harrison, 1981; Benfenati *et al.*, 1992; Ieradi *et al.*, 1996), exposure and consequent biomarker responses and heavy metal levels are relatively low in the present study, confirming emission reductions as reported by several Dutch governmental institutions. For PAHs, CCDM (2000) estimated a decreased emission by road traffic of 47.4% between 1990 and 1998. This was a net result of a decrease attributed to the introduction of cleaner combustion techniques and to an improvement in the quality of road asphalt and of an increase attributed to a growing

number of vehicle kilometers (19% between 1990 and 1998; CCDM, 2000). Still, in 1998 the estimated emission for road traffic of 159 tons PAH made up 22.1% of the total emission of 719 tons per year to the air compartment (CCDM, 2000). Differences between sites 1 and 3 in genotoxic potency and (bio)degradable Ah-receptor activity of APM (Chapter 2), however, were too small (*i.e.* less than one order of magnitude) to be reflected by the biomarker responses in the small mammals from the present study.

Concentrations of Pb and PCDD/PCDF-related biomarkers were measured in the present study as indicators for exposure to exhausts of leaded gasoline. Due to the introduction of unleaded gasoline in The Netherlands at the end of the 1980s and the ultimate ban on leaded gasoline in 1996, estimates of road traffic emissions of lead to the air compartment dramatically decreased (98.6%) in The Netherlands between 1990 and 1998 (CCDM, 2000). Consequently, Pb concentrations in kidney are no longer a useful indicator for environmental exposure to traffic emissions: average kidney concentrations were very low with ranges 0.3-2.6 and 1.1-2.1 mg Pb/kg dw for *S. araneus* and *C. glareolus*, respectively, for the different sites (Table 2). For the carnivorous shrews, much greater lead concentrations have been reported with respect to traffic emissions in the early 1980s. Chmiel and Harrison (1981), for example, reported an average concentration of 45.7 mg Pb/kg dw in kidneys from *S. araneus* sampled within 6-8 m of motorway M6 (England) with a typical traffic density of 35 000 vehicles per day. Although our sampling site 1 had a traffic density of about 93 000 vehicles/day, average Pb levels in kidneys were lower than the average background concentration of 8.6 mg/kg that Chmiel and Harrison found in shrews from their control site. Comparably, average Pb and Cd kidney concentrations in herbivorous *C. glareolus* in 1998 (present study) were, irrespective of the distance to the highway, 6-7 and 11-30 times lower than in *M. domesticus* from low- and high-traffic density sites in Rome in the early 1990s (Ieradi *et al.*, 1996).

Due to the ban on leaded gasoline, traffic emissions of PCDDs and PCDFs have been estimated to decrease with 35.3% in The Netherlands between 1990 and 1998. In 1998, traffic emission to the air compartment was estimated to be equivalent to 2.51 g TCDD, *i.e.* less than 6% of the total of 43.8 g TCDD equivalents emission to the air in The Netherlands (CCDM, 2000). This is confirmed by earlier DR-CALUX results (Chapter 2), indicating that toxic potencies of APM at sites 1 and 3 were equivalent to concentrations <1 fmol TCDD per m^3 of air.

Conclusions

All biomarkers selected in the present study yielded measurable responses, but exposure to traffic emissions did not lead to greater responses than exposure to background pollution. Greater levels of bulky aromatic DNA adducts at site 1 than at site 2 suggest that PAH exposure is greatest at site 1, but this trend (1>2) is exceeded by significantly greater DNA adduct levels at site 3, probably due to PAH input from unknown origin. Differences between biomarker responses in carnivorous shrew *S. araneus* and herbivorous vole *C. glareolus* further suggest that this input is an incidental, actual deposition of airborne pollutants rather than a historical soil pollution. Oral uptake is reasoned to be the main route of exposure to this deposition. Because DNA adduct levels increase with age, it is advisable to use only juvenile small mammals for *in situ* biomonitoring of diffuse pollution, so that interference by accumulated DNA damage and by age-dependent endogenous DNA adducts is reduced to a minimum. Kidney concentrations of Pb are no longer useful as a marker for exposure to traffic exhausts, because Pb emissions dramatically decreased since the introduction of unleaded gasoline in the 1980s. Similarly, biomarkers for PHAH exposure can no longer indicate the traffic emissions of PCDDs and PCDFs, which also dramatically decreased since the ban on leaded gasoline.

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Chapter 7

Summary and concluding remarks

Catching small mammals is a matter of fieldwork at night. Around sunset small mammal traps are set next to highway A2 in nature conservation area Autena near Everdingen (left). In the middle of the night, trapped animals are identified, registered, and caged before they are transported to the laboratory (right). Some disconnected traps can be seen in the foreground.

Summary of results

Diffuse air pollution consists of an omnipresent complex mixture of pollutants that is emitted from many widely dispersed sources and may be deposited in relatively remote areas as a result of (long-range) airborne transport. It has a heterogeneous composition in time and space and consists of many known and unknown compounds. Three objectives were defined for the present study:

1. to determine the overall exposure to diffuse air pollution by measuring biomarker responses to relevant environmental samples;
2. to determine possible (early-warning) effects of diffuse air pollution by analyzing biomarkers in ecologically relevant exposed organisms;
3. to develop a strategic research concept for a toxicological risk characterization of diffuse air pollution based on overall exposure and effect assessment.

To meet these objectives, the integrated toxic potency to affect biomarker responses has been qualified and quantified in small-scale *in vitro* bio-assays (exposure assessment) and the possible changes in biochemical and physiological endpoints have been quantified by measuring biomarkers in organisms exposed to airborne pollution in the field (effect assessment). Biomarkers were selected based on their specific response to toxicants with a specific mode of action. Selected toxicants were (1) genotoxic compounds, including PAHs, (2) arylhydrocarbon receptor (AhR) agonists including dioxin-like compounds, (3) estrogenic compounds, and (4) esterase inhibitors. Most of these toxicants were known to be relevant airborne pollutants, but the atmospheric presence of estrogens had hardly been studied yet. In all chapters, exposure or effects of diffuse air pollution have been compared between background locations and locations with notoriously exposure to traffic emissions or to pesticides. An overview of the exposure and effects assessments made in the specific chapters (indicated in parentheses) is schematically represented in Figure 1.

In **Chapter 2**, it is shown that airborne particulate matter (APM) collected next to a highway had a significantly higher genotoxic and AhR agonistic potency than APM collected in a background area located 5 km from the highway, although responses differed less than one order of magnitude. Toxic potency from APM samples collected during easterly wind conditions showed large differences among sampling days, but not among sampling sites, suggesting that long-range transport of airborne pollutants was more important than local emission under these wind conditions. Furthermore, polluted easterly wind samples had a different composition compared to less polluted samples, as could be judged from the relatively high response in the *umu*-assay in presence of a metabolic system. Trajectory

analysis demonstrated that increased genotoxicity at both locations could be attributed to APM originating from the industrial Ruhr Area in Germany.

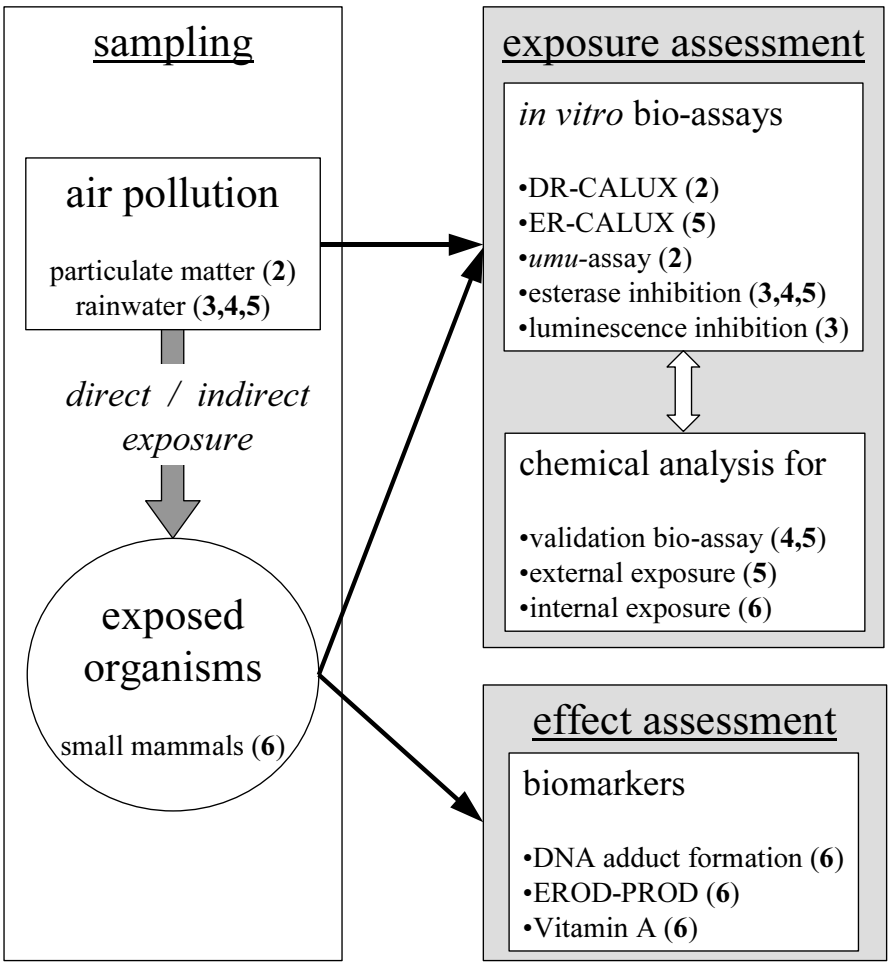

Figure 1: Schematic representation of the exposure and effects assessments made in the specific chapters (see numbers in parentheses).

Higher toxic potency of APM next to a highway (**Chapter 2**) was not reflected in biomarker responses in small mammals collected in the same highway gradient (**Chapter 6**). Although most traffic related biomarker responses were lower at 150-200 m from the highway than at 10-50 m, significantly higher responses were found in small mammals collected 5 km away from the highway at the background location of Chapter 2 with relatively clean APM. Bank voles (*Clethrionomys glareolus*) from the background location contained relatively high levels of aromatic DNA adduct levels in heart, lung and liver and lower hepatic retinyl-palmitate levels compared to voles collected closer to the highway. As these differences were not found in carnivorous common shrew (*Sorex araneus*), the relatively high biomarker responses in voles from the background location were attributed to oral exposure to an actual deposition of airborne pollutants on the vegetation.

The small mammal study described in **Chapter 6** further showed that lead levels and biomarkers for polyhalogenated aromatic hydrocarbons can no longer be used as indicators for exposure to traffic emissions. This is a result of the successful ban on the addition of lead and chlorinated scavengers to gasoline over the past decade. Finally, the study demonstrated that heterogeneity within and among sampling populations may obscure real site effects and may even lead to false conclusions if not handled properly. Especially age differences strongly affected biomarker results and should therefore be taken into account in biomarker studies with feral small mammals.

Chapter 3 describes the development and optimization of a small-scale bio-assay for measuring toxic potencies of organophosphate and carbamate insecticides in rainwater. This bio-assay is based on the capacity of these insecticides to block the enzyme acetylcholinesterase (AChE), thereby causing an inhibition of the hydrolysis of the neurotransmitter acetylcholine. Hydrolysis of the non-specific substrate N-methylindoxylacetate (MIA) by carboxylesterases from a crude homogenate of honeybee heads appeared to be much more sensitive to inhibition by organophosphate insecticides than the commonly measured hydrolysis of acetylthiocholine (ATC) by purified AChE. The higher sensitivity of carboxylesterases is attributed to the instant formation of a reversible Michaelis-Menten complex with the inhibitor at concentrations where AChE is not inhibited yet. Because of this greater sensitivity, the optimized bio-assay was very suitable for measuring the esterase inhibiting potency of rainwater extracts (**Chapters 3, 4 and 5**). Dichlorvos was used as a model organophosphate inhibitor allowing quantification of the esterase inhibiting potency of rainwater samples into a dichlorvos equivalent (DEQ) concentration.

In a pilot study with rainwater samples collected on two sites in the Province of South-Holland over four consecutive 14-day periods (**Chapter 4**), good correspondence was observed between measured esterase inhibiting potency and the toxic potency calculated from chemically analyzed concentrations of organophosphate and carbamate insecticides. The additional value of bio-assays to chemical analyses was demonstrated by the fact that the measured toxic potency was on average about 50% higher than the toxic potency calculated from chemical analyses. This difference between the calculated and measured toxic potency may be explained by the fact that the calculated toxic potency does not account for (1) pesticides present at concentrations below the chemical detection limit, (2) non-analyzed pesticides and metabolites, and (3) pesticides for which insufficient toxicity data are available. Compared to a relative background site, the esterase-inhibiting potency of rainwater from a site with intense horticultural activities (greenhouses) was at maximum 6 times higher.

Extremely high esterase inhibiting potency was found in a sample from the greenhouse area. This sample contained high concentrations of dichlorvos, pirimiphos-methyl, mevinphos, and methiocarb, with the first two compounds exceeding EC_{50}-values reported for Daphnia. The esterase inhibiting potency of this particular sample was equivalent to a dichlorvos concentration that exceeded the maximum permissible concentration (MPC) set for dichlorvos in surface water almost by a factor 2000. This sample also gave the highest response in another specially adapted bio-assay for general toxicity with bioluminescent *Vibrio fischeri* bacteria, although this response is not specific for pesticides.

The esterase inhibiting potency as well as the pesticide composition of rainwater were subsequently measured in a year-round monitoring program on three locations in the Province of South-Holland (**Chapter 5**). The agricultural activities at the chosen sites were typical for greenhouse horticulture, flower bulb cultivation and for a background situation. Esterase inhibiting potency of rainwater significantly depended on sampling location, with greatest responses found at the horticultural site. Principal component analysis confirmed that esterase inhibiting potency significantly correlated with analyzed concentrations of organophosphate and carbamate pesticides. Maximum concentrations of individually analyzed pesticides and esterase inhibiting potencies expressed as dichlorvos equivalent concentrations in 1998 were lower than in the pilot study of 1997 (**Chapter 4**), but incidentally still exceeded MPCs for surface water and Daphnia EC_{50}-values.

The estrogenic potency of the rainwater samples significantly varied with sampling season, with greatest responses found in spring (**Chapter 5**). Local emission was less important for this parameter, suggesting that pseudo-estrogens have other and more dispersed sources than esterase inhibitors. Although estrogenic potency significantly correlated with organochlorine (OC) pesticide concentrations, this does not automatically imply a causal relationship. Estrogenic potency of the measured OC compounds could not explain the estrogenic potency of the rainwater samples. Co-occurrence of OC pesticides with estrogenic compounds seems to be the most obvious explanation. These compounds may be related to OC pesticide use or just have a comparable environmental distribution. For instance, pseudo-estrogens nonylphenol-ethoxylates and bisphenol A have been found in rainwater. The first are used as additives in pesticide formulations but there are no records of bisphenol A use in pesticide formulations. Incidentally, the estradiol equivalent concentrations in rainwater samples exceeded the lowest observed effect levels of estrogens reported in literature for vitellogenesis in male rainbow trout. As rainwater pollutants are generally very diluted and adsorbed to sediments, suspended matter, and plants when deposited in surface water, it is

unlikely that the levels of estrogenic and esterase-inhibiting potency pose a direct risk for aquatic ecosystems. Possibly, shallow and mainly rainwater-fed water pools form an exception to this rule.

Concluding remarks

Proposed concept

The previous chapters summarized above have mainly focused on the first two objectives defined for the present study, *i.e.* to make an exposure and an effect assessment for diffuse air pollution by measuring biomarkers in *in vitro* bio-assays and in *in vivo* exposed organisms. The third objective of the present study is addressed here: a strategic research concept is proposed for risk characterization of diffuse air pollution. The basis of the proposed approach is concluded from the experiences of the previous chapters. It is characterized by alternating applications of biological and chemical analyses (Figure 2), yielding a better understanding of the composition, the character and the (ecological) consequences of the toxic potency of the pollution. Figure 2 presents a flow scheme leading to a risk characterization of diffuse air pollution. It shows the main steps to take, some of which have been elaborated more extensively in the present thesis than others.

The first step is to collect samples that are relevant for the type of diffuse air pollution under study. Samples of air pollution can be collected from gaseous phase, APM and/or rainwater, depending on the solubility and the volatility of the atmospheric pollutants expected to be responsible for the toxic potency. Many compounds distribute over two or three of the possible phases present in the air, and should thus be sampled in different atmospheric phases. In addition, sampling should be considered of food sources that have been exposed to (deposited) airborne pollution when making a hazard characterization for sentinel species.

The second step is sample processing, which is necessary because the original matrix of the crude environmental samples cannot directly be administered to the enzymes (*e.g.* esterase-inhibition assay), bacteria (*e.g. umu*-assay) or cells (*e.g.* CALUX-assays) of the *in vitro* bio-assays. Extraction, subsequent clean-up, and concentration of the sample is often required as the crude sample contains components that can disturb the measurement, is non-sterile, or contains concentrations below detection limit. For pollutants dissolved in the aqueous phase or adsorbed to the solid APM phase, extraction procedures can be adopted from analytical chemistry that have been optimized for application in bio-assays (Chapters

2-5). However, no such procedures are available to extract compounds from the gaseous phase and add them to the bio-assay in a quantifiable manner. Reliable quantification of the toxic potency of volatile compounds is seriously hampered, because they evaporate immediately directly from the extract or during solvent change. This is a shortcoming that bio-assays share with chemical analyses.

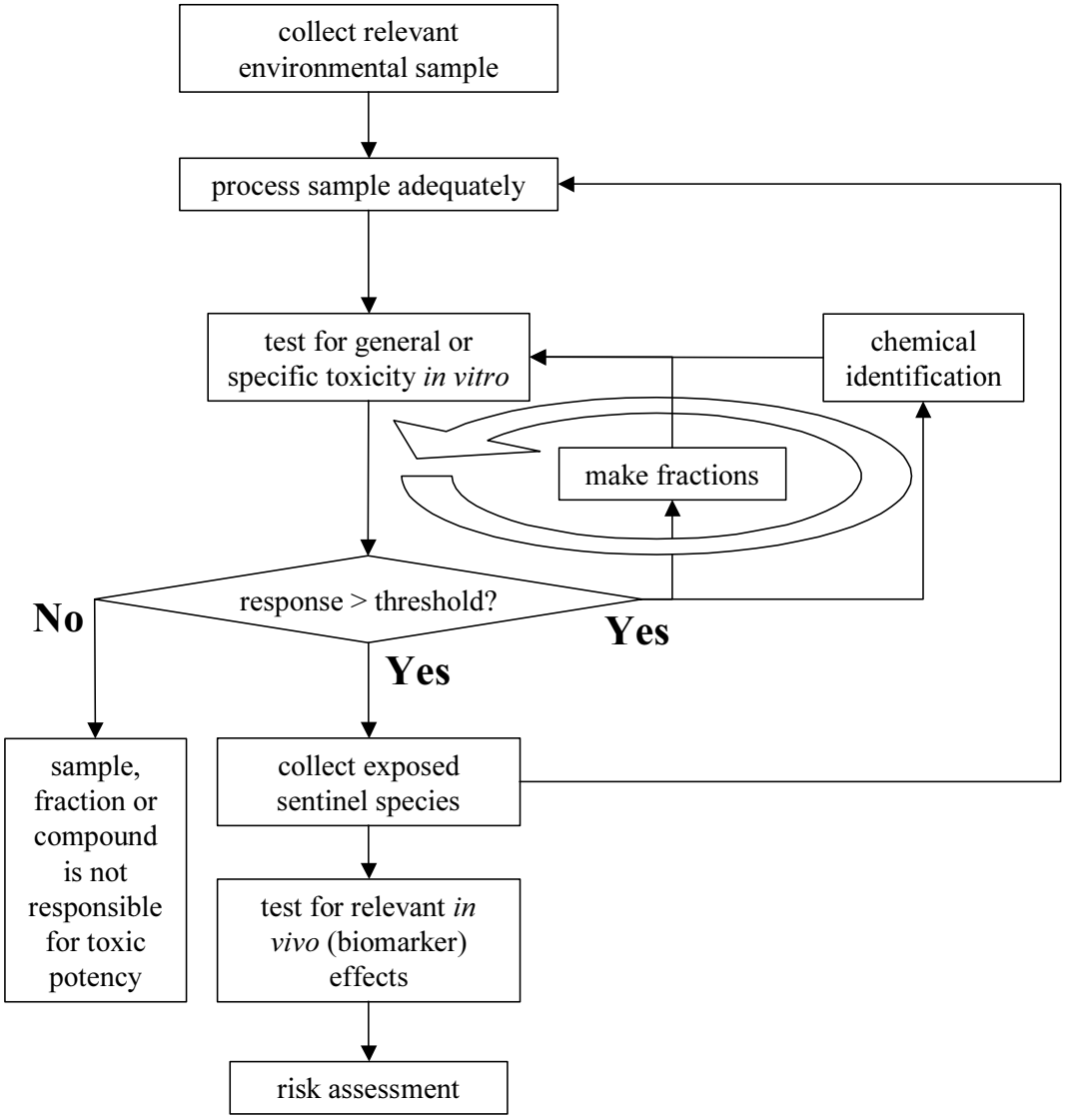

Figure 2: Proposed strategic research concept for characterization of the toxic potency of atmospheric pollution and subsequent hazard assessment.

With bio-assays, sample processing may lead to introduction of extra toxicity or a background signal. For instance, APM was sampled on quartz fiber filters (Chapter 2) because extracts from glass fiber filters appeared to be cytotoxic to DR-CALUX cells. Similarly, processing of samples for the ER-CALUX was performed using laboratory devices

made from Teflon or preferably from glass (Chapter 5), because many plastics leak plasticizers with estrogenic activity to the samples. In conclusion, environmental sampling and subsequent sample processing are very critical steps in applying bio-assays for hazard characterization.

Upon adequately processing, environmental samples can be tested in specific or non-specific bio-assays (Chapter 1). Hazard assessment can then be made by comparing the bio-assay responses to threshold exposure levels for toxic effects:

1. Bio-assay responses can be compared to effect levels measured in *in vivo* studies with single model compounds. For instance, estradiol equivalent (EEQ) concentrations in rainwater were compared to EEQ effect levels on vitellogenesis in male rainbow trout, obtained from the literature (Chapter 5).
2. Bio-assay responses can be compared to environmental criteria derived from *in vivo* studies with single model compounds. For instance, dichlorvos equivalent (DEQ) concentrations were compared to maximum permissible concentrations set for dichlorvos in surface water in The Netherlands (Chapters 4 and 5).
3. Bio-assay responses can mutually be compared between samples from supposedly clean and notoriously exposed locations so that background pollution can be compared to a worst-case situation. For instance, APM collected in a background location and next to a busy highway were mutually compared with respect to their genotoxic and AhR agonistic potency (Chapter 2).

Of course, the main route of exposure under field conditions may differ from the route for which threshold levels have been derived. For instance, the toxic potency of APM determined in Chapter 2, should not directly be compared to critical airborne concentrations of (geno-)toxicants such as the outdoor maximum permissible concentration of 1 ng/m^3 set for benzo[a]pyrene (VROM, 1999), because this critical concentration was derived for human inhalatory exposure, whereas results from Chapter 6 suggest that oral uptake of deposited APM is a more important route of exposure to DNA binding air pollution for herbivorous small mammals. This example illustrates that the route of exposure should be taken into account when using threshold levels to evaluate the hazard of the toxic potency determined in bio-assays.

Obviously, identification of the compounds that contribute most to the toxic potency of the environmental sample is often desired. For this purpose, a Toxicity Identification and Evaluation (TIE) procedure is the most suitable approach. Samples exceeding threshold levels are separated into several different fractions and tested again in the bio-assay. Preferably, this

step is repeated several times to reduce the number of non-active compounds. The active fractions are then chemically analyzed, which is a difficult step implying searching for unknown compounds. Finally, the identified compounds are tested for their toxic potency and their contribution to the overall toxic potency of the sample is determined.

Bio-assay responses exceeding threshold values for a relevant route of external exposure indicate that the measured levels of diffuse pollutants may pose a hazard to man and ecosystem. For a complete risk characterization, further *in vivo* studies can then be applied to determine internal exposure and subsequent effects. For instance, feral (Chapter 6) or laboratory animals can be exposed *in situ* to the environmental mix of pollutants present in the field. Alternatively, feral or laboratory animals can be exposed by a relevant route of exposure in the laboratory (*ex situ*) to the environmental samples exceeding the bio-assay threshold, to the most potent fractions thereof, or to the individual toxic compounds identified in the TIE-procedure.

To assess internal exposure, tissue samples from *in vivo* exposed organisms can be analyzed in *in vitro* bio-assays comparably to environmental samples. For effect assessment, (biomarker) effects should be measured in the exposed organisms. Preferably, selected biomarkers should be highly indicative for adverse effects to enable risk characterization higher than the individual level (Walker *et al.*, 2001).

Finally, a risk characterization can be made based on an interpretation of the toxic potency measured in the bio-assays, the chemical characterization of the active compounds, and the *in vivo* effects found after exposure in the laboratory and/or in the field.

Conclusions

As far as the proposed approach has been applied in the present study, the following conclusions are drawn for the selected groups of airborne pollutants:

- Airborne particulate matter (APM) at background locations contains genotoxic and AhR agonistic potency. Background levels are significantly lower than next to a very busy highway, but differences between sites are amply within one order of magnitude. Trajectory analyses attribute incidentally high toxic potencies and deviating toxic profiles with easterly wind to airborne pollutants originating from the industrial German Ruhr Area by long-range atmospheric transport.
- Airborne AhR agonistic potency is completely attributable to readily biodegradable compounds such as PAHs, and not to stable congeners such as polychlorinated dioxins and furans. This result proves that remediation of waste incinerators and banning the use

of leaded gasoline with halogenated scavengers have been successful measures to reduce airborne levels of these compounds. Similarly, the ban on leaded gasoline has resulted in dramatically decreased internal lead levels in animals living next to a highway, which can no longer be used as a biomarker for exposure to traffic emissions. Lead emitted by traffic can no longer be considered as an environmental problem.

- The fact that genotoxic and AhR agonistic potencies in APM decrease with increasing distance to the highway is not reflected in *in vivo* biomarkers in feral small mammals. Apparently, exposure to traffic emissions does not pose a risk to the selected species. Actually, *in vivo* biomarker responses suggest that small mammals at the background location are exposed to higher levels of pollutants, probably due to an actual deposition of airborne pollutants on the vegetation, which is readily available for herbivorous animals such as voles.
- Esterase-inhibiting potency in rainwater varies with sampling location and regularly reaches levels equivalent to dichlorvos concentrations that exceed environmental criteria set for surface water, especially in horticultural areas. Estrogenic potency of rainwater is less related to sampling site, suggesting that it originates from more dispersed sources. Estradiol equivalent concentrations in rainwater incidentally exceed effect concentrations for fish. The ecological consequences of these deposited toxic potencies are unknown yet, but for remote and mainly rainwater-fed ecosystems they may be a significant input.

With respect to the applied biomarker methodology, the following conclusions are drawn:

- Bio-assays are valuable tools for hazard characterization of complex mixtures of diffuse air pollutants. In general, toxic potencies measured in bio-assays are higher than can be explained based on chemical analyses. Many compounds and their metabolites contributing to the toxic potency have probably not been analyzed because their chemical identity is unknown, their concentrations are below the analytical detection limit, or their contribution to the overall toxic potency is not recognized.
- For hazard characterization, alternating applications of biological and chemical analyses are proposed (Toxicity Identification and Evaluation [TIE] approach) to get a better understanding of the composition, the character and the (ecological) consequences of the toxic potency of the pollution.
- Hydrolysis of the non-specific substrate N-methylindoxylacetate by carboxylesterases from a crude homogenate of honeybee heads is much more sensitive to inhibition by

organophosphate insecticides than the commonly measured hydrolysis of acetylthiocholine by purified acetylcholinesterase. The higher sensitivity of carboxylesterases is attributed to instant reversible inhibition at inhibitor concentrations where acetylcholinesterase is not yet inhibited. This enables detection of low levels of esterase inhibitors in rainwater.

- Heterogeneity within and among sampling populations should be taken into account when using small mammals for environmental monitoring. Especially differences in age and to a lesser extent in sex composition of the sampling populations can obscure biomarker results and may lead to false conclusions.
- Although the selected species do not seem to be affected by traffic emissions, it cannot be excluded that exposure over the last 50 years has led to a selection of resistant species while sensitive species already have disappeared.

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Samenvatting

Two major types of diffuse air pollutants studied in this thesis: agricultural pesticide emissions (top) and traffic emissions (bottom)

Sources: JC van de Zande (IMAG; top) and F Vera (KLPD Driebergen; bottom)

In Nederland worden verreweg de meeste milieuverontreinigende stoffen uitgestoten naar het compartiment lucht. Zo is de omvang van de uitstoot van organische verbindingen naar lucht ongeveer 10 keer groter dan naar water en 300 keer groter dan naar de bodem. Veel van de luchtverontreinigende stoffen zijn afkomstig uit verstrooide bronnen, zoals verkeer, industrieën, huishoudens en landbouw. Door atmosferisch transport kan luchtverontreiniging relatief afgelegen gebieden bereiken die niet in de buurt liggen van notoire bronnen, zoals snelwegen of industriegebieden. Hierdoor worden mens en milieu in Nederland overal en altijd blootgesteld aan een grauwsluier van luchtverontreiniging, die bestaat uit een complex mengsel van duizenden stoffen uit nationale en internationale bronnen. Dit diffuse mengsel verandert van samenstelling in ruimte en tijd en bevat een substantiële fractie stoffen met een onbekende chemische identiteit en giftigheid.

Het doel van het onderzoek beschreven in dit proefschrift was drieledig:

1. de blootstelling aan deze diffuse verontreiniging karakteriseren,
2. de mogelijke effecten van deze blootstelling inventariseren,
3. een strategisch concept ontwikkelen om te komen tot een toxicologische risicobeoordeling van diffuse luchtverontreiniging.

Om de eerste twee doelen te verwezenlijken is gebruik gemaakt van een benadering met biomarkers. Biomarkers zijn veranderingen in cellulaire of biochemische processen, structuren of functies die worden veroorzaakt door blootstelling aan niet-natuurlijke (niveaus van) stoffen en gemeten kunnen worden in biologische systemen of monsters. De **blootstelling** aan diffuse verontreiniging is gekarakteriseerd door dit soort veranderingen te meten in laboratoriumtesten (bio-assays), waarin cellen, bacteriën of enzymen *in vitro* zijn blootgesteld aan milieumonsters uit het veld. De mogelijke **effecten** zijn geïnventariseerd door dit soort veranderingen te meten in organismen die onder veldomstandigheden zijn blootgesteld aan diffuse verontreiniging.

Er is gebruik gemaakt van zowel specifieke als aspecifieke biomarkers. Een specifieke biomarker is indicatief voor blootstelling aan stofgroepen met hetzelfde specifieke werkingsmechanisme, terwijl een aspecifieke biomarker een indruk geeft van algemene toxiciteit die wordt gekenmerkt door één specifiek toxicologisch werkingsmechanisme. In dit proefschrift is gebruik gemaakt van specifieke biomarkers die indicatief zijn voor vier verschillende stofgroepen (zie Hoofdstuk 1, Figuur 1):

1. genotoxische (dwz DNA-beschadigende) stoffen zoals polycyclische aromatische koolwaterstoffen (PAKs);

2. stoffen die de dioxine-receptor (AhR) kunnen activeren, zoals persistente polychloordibenzo-*p*-dioxines (PCDDs) en polychloor-bifenylen (PCBs), maar ook de meer biologisch afbreekbare PAKs;
3. oestrogene stoffen die het vrouwelijk geslachtshormoon oestradiol kunnen nabootsen zoals sommige weekmakers, grondstoffen voor plastics, reinigingsmiddelen en organochloor bestrijdingsmiddelen;
4. esterase-remmende stoffen zoals organofosfaat en carbamaat insecticiden.

De meeste van deze stofgroepen zijn gekozen omdat ze een prominente bijdrage leveren aan de diffuse luchtverontreiniging. Oestrogene stoffen zijn gekozen omdat hun atmosferische aanwezigheid tot nu toe amper bestudeerd is en hun aanwezigheid wel wordt vermoed.

Omdat er binnen Nederland zeer waarschijnlijk geen 'controle' gebieden bestaan zonder diffuse luchtverontreiniging, zijn blootstellingen en mogelijke effecten vergeleken tussen relatief afgelegen gebieden (achtergrondsituatie) en gebieden in de nabijheid van bronnen van luchtverontreiniging ('worst case'). Gelet op de geselecteerde stofgroepen zijn een drukke snelweg en enkele gebieden met intensieve tuinbouw als worst case situaties gekozen.

De toxische potentie van genotoxische en AhR activerende luchtverontreiniging in het relatief afgelegen natuurgebied De Regulieren nabij Culemborg en vlak langs de snelweg A2 ter hoogte van Everdingen is bepaald in monsters van zwevende deeltjes in de lucht (airborne particulate matter: APM). Hierbij is gebruik gemaakt van twee verschillende specifieke bio-assays, respectievelijk de *umu* en de DR-CALUX assay (**Hoofdstuk 2**). De toxische potentie van APM verzameld vlak langs de snelweg was significant hoger dan in het natuurgebied, maar de verschillen waren relatief klein (< factor 10). Tijdens oostenwind waren de verschillen tussen de twee monsterpunten nog kleiner, maar tussen de monsterdagen groter, hetgeen betekent dat lokale uitstoot bij deze windrichting minder belangrijk is dan lange afstand transport van luchtverontreiniging. Bovendien bevatten oostenwind monsters met een hoge toxische potentie een afwijkende mix van stoffen, aangezien ze een hogere respons gaven na metabole omzetting dan (oostenwind) monsters met een relatief lage toxische potentie. Analyse van de trajecten die de bemonsterde luchtpakketten hebben afgelegd, toonde aan dat verhoogde genotoxiciteit tijdens oostenwind kan worden toegeschreven aan APM afkomstig van het industriële Roergebied in Duitsland. Overigens kon op beide monsterlocaties de totale AhR-activerende potentie van APM aan PAKs en verwante aromatische verbindingen worden toegeschreven, en werd er geen persistente dioxine-achtige activiteit in het APM gevonden (**Hoofdstuk 2**). Dit resultaat toont aan dat de sanering van vuilverbrandingsinstallaties een succesvolle maatregel is geweest om de uitstoot van

gechloreerde dioxines en furanen naar de lucht te verminderen. Ook het verbod op loodhoudende benzine waaraan chloorhoudende stoffen werden toegevoegd heeft geleid tot een verminderde uitstoot van dioxines en furanen naar de lucht.

De verschillen in toxische potentie van APM tussen beide gebieden kwamen niet overeen met de resultaten van de biomarkers gemeten in kleine zoogdieren (bosspitsmuizen en rosse woelmuizen; **Hoofdstuk 6**). Ofschoon de meeste biomarkers een lagere blootstelling aan verkeersgerelateerde stoffen toonden op 150-200 m van de snelweg vergeleken met vlak langs de snelweg (10-50 m), waren biomarker responsies het hoogst in De Regulieren gelegen op 5 km van de snelweg. Rosse woelmuizen (*Clethrionomys glareolus*) uit dit natuurgebied hadden hogere niveaus van DNA adducten in hart, long en lever en lagere voorraden vitamine A in de lever dan rosse woelmuizen die nabij de snelweg werden gevangen. Omdat deze verschillen in veel mindere mate werd gevonden in carnivore bosspitsmuizen (*Sorex araneus*) dan in overwegend herbivore rosse woelmuizen, zijn de verhoogde biomarkers in het natuurgebied toegeschreven aan recente lokale depositie van luchtverontreiniging op de vegetatie (**Hoofdstuk 6**). Deze studie bevestigde verder dat het verbod op loodhoudende benzine niet alleen succesvol is geweest om de verkeersuitstoot van dioxine-achtige stoffen terug te dringen, maar ook die van lood. Bovendien bleek dat bij het analyseren van biomarker-resultaten rekening moet worden gehouden met leeftijds- en geslachtsverschillen tussen individuen uit verschillende monsterpopulaties om te voorkomen dat verkeerde conclusies worden getrokken (**Hoofdstuk 6**).

Omdat veel bestrijdingsmiddelen die in de atmosfeer voorkomen uit de lucht worden uitgeloogd door natte depositie, is de esterase-remmende en de oestrogene potentie bepaald van regenwater afkomstig uit gebieden met laag en hoog pesticidengebruik. Om de toxische potentie van organofosfaat (OP) en carbamaat (CARB) insecticiden in regenwater te kunnen kwantificeren is een gevoelige bio-assay ontwikkeld, gebaseerd op het werkingsmechanisme van deze bestrijdingsmiddelen om acetylcholinesterase en andere enzymen met esterase activiteit te blokkeren (**Hoofdstuk 3**). Om de toxische potentie van OP bestrijdingsmiddelen te meten bleek de substraat-enzym combinatie van N-methylindoxylacetaat en carboxylesterases uit homogenaat van bijenkoppen (*Apis mellifera*) gevoeliger dan de vaak gebruikte combinatie van acetylthiocholine en puur acetylcholinesterase. Op basis van deze hogere gevoeligheid is de bio-assay geoptimaliseerd en vervolgens toegepast voor meting van de esterase-remmende potentie in regenwater, uitgedrukt als equivalent concentraties van het model OP bestrijdingsmiddel dichloorvos (**Hoofdstukken 3, 4 en 5**).

De esterase-remmende potentie van regenwater bleek goed overeen te komen met de geanalyseerde concentraties van individuele OP en CARB insecticiden (**Hoofdstuk 4**). De gemeten toxische potentie is echter wel 50% hoger dan de toxische potentie die berekend kan worden op basis van de concentraties van de bekende individuele stoffen. Het feit dat de toxische potentie gemeten in de bio-assay hoger is dan de berekende toxische potentie op basis van chemische analyses, wijst erop dat sommige stoffen worden gemist in de berekening omdat (1) ze voorkomen in concentraties kleiner dan de detectielimiet, (2) ze niet geanalyseerd zijn, en (3) onvoldoende toxiciteitsgegevens van deze stoffen bekend zijn. Vergeleken met regenwatermonsters afkomstig uit een achtergrondlocatie gelegen aan de Nieuwkoopse Plassen (Noorden) was de gemeten esterase-remmende potentie van regenwatermonsters uit het kassengebied in het Westland (Naaldwijk) maximaal zes keer hoger. Het meest vervuilde monster werd verzameld in het kassengebied. De gemeten esterase-remmende potentie van dit monster was 2000 keer hoger dan de Nederlandse norm voor dichloorvos in oppervlaktewater. Dit monster was ook het meest toxisch in de speciaal aangepaste aspecifieke bio-assay voor algemene toxiciteit waarin gebruik wordt gemaakt van lichtgevende *Vibrio fischeri* bacteriën (**Hoofdstuk 4**).

Op basis van de geslaagde oriënterende proef (pilot; **Hoofdstuk 4**) is vervolgens de esterase-remmende potentie van regenwater een jaar lang (1998) bepaald op drie locaties in de provincie Zuid-Holland (**Hoofdstuk 5**). Esterase-remmende potentie bleek significant afhankelijk te zijn van de monsterlocatie, waarbij regenwater uit de kasgebieden in het Westland (Naaldwijk) hogere esterase-remmende potentie hadden dan regenwater uit de bollenstreek (Hillegom) of uit de achtergrondlocatie gelegen aan de Nieuwkoopse Plassen (Noorden). Met principale component analyse (PCA) kon worden aangetoond dat er een significante correlatie bestond tussen de esterase-remmende potentie in de regenwatermonsters en concentraties van individuele OP en CARB bestrijdingsmiddelen. Ofschoon de hoogste concentraties en toxische potenties lager waren dan in de pilotstudie (**Hoofdstuk 4**), werden incidenteel niveaus gevonden die hoger waren dan Nederlandse normen voor oppervlaktewater en EC_{50}-concentraties voor watervlooien.

Van dezelfde regenwatermonsters uit 1998 is ook de oestrogene potentie bepaald (**Hoofdstuk 5**). Deze bleek niet significant gerelateerd aan de monsterlocatie, maar wel aan het monsterseizoen: de hoogste oestrogene potentie werd gevonden in het voorjaar. Ofschoon de oestrogene potentie correleerde met de aanwezigheid van organochloor (OC) bestrijdingsmiddelen in het regenwater, waren de OC concentraties te laag om de gemeten potentie te kunnen verklaren. Kennelijk wordt de oestrogene potentie van het regenwater

veroorzaakt door stoffen die gerelateerd zijn aan het gebruik van OC bestrijdingsmiddelen of een hiermee vergelijkbare verspreiding in het milieu hebben. Incidenteel overschreed de gemeten oestrogene potentie de laagste effect concentratie (LOEC) die voor vissen wordt gerapporteerd in de literatuur. Aangezien regenwater in het algemeen sterk verdund wordt bij neerslag in oppervlaktewater, is het onwaarschijnlijk dat de oestrogene en esterase remmende potentie een direct risico vormen voor aquatische ecosystemen, wellicht met uitzondering van ondiepe en relatief afgelegen vennetjes, waarvoor vervuild regenwater mogelijk juist een belangrijke input van toxische potentie kan vormen.

In de **Hoofdstukken 2 tm 6** zijn studies beschreven waarin biomarkers zijn gebruikt voor het karakteriseren van de blootstelling aan diffuse verontreiniging (eerste doel) en het inventariseren van de mogelijke effecten van deze blootstelling (tweede doel). Op basis van de ervaringen uit deze studies, is in **Hoofdstuk 7** een strategisch concept voorgesteld voor een toxicologische risicobeoordeling (derde doel). De voorgestelde strategie kent een aantal stappen (zie Hoofdstuk 7, Figuur 2), die inzicht geven in de toxische potentie van diffuse luchtverontreiniging, de identiteit en fysisch/chemische eigenschappen van de actieve componenten, en de mogelijke effecten van diffuse luchtverontreiniging. Allereerst worden relevante milieumonsters verzameld en zodanig opgewerkt dat hun toxische potentie in een bio-assay bepaald kan worden. Vervolgens wordt de gemeten toxische potentie getoetst aan drempelwaarden door de bio-assay resultaten te vergelijken met (1) effect concentraties van modelstoffen, (2) milieunormen voor modelstoffen of (3) bio-assay resultaten van milieumonsters verzameld in zeer schone of notoir vervuilde gebieden. Voor het identificeren van de stoffen die de hoogste bijdrage leveren aan de toxische potentie wordt een zogenaamde Toxiciteit Identificatie en Evaluatie (TIE) procedure voorgesteld, waarin monsters die drempelwaarden overschrijden op basis van hun fysisch-chemische eigenschappen worden opgesplitst in fracties die opnieuw worden getest in de bio-assay. De actieve fractie wordt vervolgens chemisch geanalyseerd en de bijdrage van de geïdentificeerde stoffen aan de totale toxische potentie wordt bepaald door deze individueel in de bio-assay te testen. Diffuse luchtverontreiniging met een toxische potentie die de grenswaarde overschrijdt, vormt mogelijk een risico voor mens en/of milieu. Om dit risico te inventariseren, worden biomarkers met een hoge voorspellende waarde voor negatieve effecten gemeten in wilde dieren die onder natuurlijke omstandigheden passief zijn blootgesteld aan de mix van stoffen in het veld (*in situ*). Biomarkers kunnen ook worden gemeten in dieren die in het laboratorium (*ex situ*) actief zijn blootgesteld aan milieumonsters die drempelwaarden overschrijden, of aan actieve fracties of componenten van deze monsters.

Dit proefschrift bevestigt dat bio-assays waardevolle methodes zijn om de blootstelling aan diffuse verontreining te kwalificeren en te kwantificeren. In het algemeen is de toxische potentie gemeten in een bio-assay hoger dan kan worden verklaard op basis van chemische analyses, omdat een substantiële fractie van het complexe mengsel wel bijdraagt aan de toxische potentie maar een onbekende identiteit, concentratie en toxiciteit heeft. Diffuse luchtverontreiniging met alle vier de gekozen stofgroepen is overtuigend aangetoond in alle bemonsterde locaties, wat het alom aanwezige karakter van deze verontreiniging bevestigt. De oestrogene potentie verschilt niet tussen de bemonsterde locaties. Voor de overige stofgroepen wordt weliswaar lagere toxische potenties gemeten in achtergrondgebieden dan in notoir blootgestelde gebieden, maar de verschillen zijn klein en altijd minder dan een factor 10. Voor zover getest, zijn deze verschillen ook te klein om te worden weerspiegeld in biomarkers in kleine zoogdieren die *in situ* zijn blootgesteld aan diffuse luchtverontreiniging.

Curriculum vitae

Timo Hamers was born in Maastricht on January 26, 1968. After graduation from Sint Maartenscollege in 1986 (VWO-B), he studied Environmental Hygiene at Wageningen Agricultural University. He took part in two graduation projects: one at the Toxicology Group (Dr Ir GAJM Jagers op Akkerhuis; Prof Dr JH Koeman) and one at the Aquatic Ecology Group (Dr RMM Royackers). His practical period was fulfilled at The Institute of Wildlife and Environmental Toxicology (TIWET) in Pendleton, SC (Dr L Brewer; Dr RJ Kendall). After passing MSc exam with credit in 1992, he started working as a desk editor for the EUROSOL proceedings at the Netherlands Integrated Soil Research Program, Wageningen (Prof Dr HJP Eijsackers). In 1993 he worked as an international guest worker at the Soil Department of the National Environmental Research Institute in Silkeborg, DK (Dr PH Krogh; Dr H Løkke). From 1993 to 1996, he carried out three different projects at the Laboratory of Ecotoxicology at the National Institute of Public Health and the Environment (RIVM) in Bilthoven (Dr J Notenboom, Dr D van de Meent). From 1996 onwards, he has been working as a scientific researcher at the Toxicology Group of Wageningen University investigating the integrated exposure of natural ecosystems with airborne xenobiotic compounds (Dr AJ Murk; Prof Dr JH Koeman). Results of this project that was funded by the Dutch Ministry of Agriculture, Nature Conservation and Fisheries (LNV) have been described in the present thesis. Since 2001, he performs a two-year research project on the toxicological evaluation of diffuse pollutants in the Biesbosch area, as part of the NWO Stimulation Program System-Oriented Ecotoxicological Research (SSEO). In January 2003 he will start a new position as project leader ecotoxicology at the Institute for Environmental Studies (IVM) in Amsterdam.

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Main abbreviations

2-AA:	2-aminoanthracene
ACh:	acetylcholine
AChE:	acetylcholinesterase
AhR:	arylhydrocarbon receptor
APM:	airborne particulate matter
ATC:	acetylthiocholine
BaP:	benzo[a]pyrene
BEQ:	BaP equivalent
BPA:	bisphenol A
CARB:	carbamate
CYP:	cytochrome P450
DEQ:	dichlorvos equivalent
DL:	detection limit
DR-CALUX:	dioxin-responsive chemical-activated luciferase gene expression
DRE:	dioxin-responsive element
DTNB:	dithiobisnitrobenzoic acid (Ellman's reagens)
EC_{50}:	median effect concentration
EEF:	estrogen equivalency factor
EEQ:	estradiol equivalent
ER:	estrogen receptor
ER-CALUX:	estrogen receptor mediated chemical activated luciferase gene expression
ERE:	estrogen receptor element
EROD:	ethoxyresorufine-O-dealkylation
LC_{50}:	median lethal concentration
LD_{50}:	median lethal dose
LOEC:	lowest observed effect concentration
LoQ:	limit of quantification
MIA:	N-methylindoxyl acetate
MPC:	maximum permissible concentration
NOEC:	no observed effect concentration
4-NQO:	4-nitroquinolin-oxid
OC:	organochlorine
OP:	organophosphate
PAHs:	polycyclic aromatic hydrocarbons
PBDEs:	polybrominated diphenyl ethers
PCA:	principal component analysis
PCBs:	polychlorinated biphenyls
PCDDs:	polychlorinated dibenzo-*p*-dioxins
PCDFs:	polychlorinated dibenzofurans
PHAHs:	polyhalogenated aromatic hydrocarbons
PNEC:	predicted no effect concentration
PROD:	pentoxyresorufine-O-dealkylation
RE:	retinol
RP:	retinyl-palmitate
TCDD:	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEQ:	TCDD equivalent
TIE:	toxicity identification and evaluation
VTG:	vitellogenin

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Dino