Developmental disorders induced by pesticide degradation products
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General Introduction

Current intensification of agriculture in Kenya and other developing countries demands increased pesticide use, which may lead to pesticide contamination of ground and surface water. It has been estimated that less than 0.1% of the pesticides applied to crops reach the target pests, thus more than 99% of the applied pesticides have a potential to impact non-target organisms (Albert et al. 1992). In the tropics, high levels of ultra-violet radiations, high temperatures and high rainfall with subsequent runoffs modify these risks (Bossan et al. 1995; Abdullah et al. 1997).

Pesticides degrade in the environment into transformation products that could alleviate, enhance and/or increase the diversity of their toxic effects (Kraak et al. 1997; Bleeker et al. 1999; Admiraal et al. 2000). Besides the acute toxic effects, teratogenic, genotoxic, mutagenic, carcinogenic and other subtle toxic effects are important probable endpoints, which may differ between the parent compounds and their degradation products. However, ecotoxicological studies of pesticides in the aquatic environment have hitherto underrated their degradation products. The invariable persistence of the degradation products underlines the need to study their long-term effects in the environment. Most knowledge of pesticides is derived from studies in the temperate regions. The tropical environment, which is the focus of this thesis, has been sparsely studied, in spite of the disparate physical and chemical environmental conditions prevailing there.

Based on their extensive usage in Kenya to control weed in the cereal crops and tick in the dairy industry, chloroacetanilides, formamidines and their resulting environmentally stable anilines degradation products are studied for their acute toxic, genotoxic, and teratogenic effects on the ubiquitous Chironomus riparius, Vibrio fischeri and the locally abundant Xenopus laevis.

Chloroacetanilides, formamidines and their degradation products

The chloroacetanilides alachlor, butachlor (synthesized from 2,6-diethylaniline), metolachlor and acetochlor (derived from 2-ethyl-6-methylaniline) are some of the most intensively used herbicides worldwide
General Introduction

(Hill et al. 1997), while the formamidine amitraz (derived from 2,4-dimethylaniline) is an important acaricide used extensively in tick endemic areas of Australia and Southern Africa including Kenya (Partow 1995; Baxter and Barker 1999). The use of amitraz in Kenya is currently on the increase to alleviate the problems of increased tick resistance to alternate acaricides.

In the environment the chloroacetanilides revert to the precursor anilines as the environmentally stable degradation products (Tiedje and Hagedorn 1975; Allcock and Woods 1978; Kimmel et al. 1986; Knowles and Hamed 1989; Wei and Vossbrinck 1992; Hill et al. 1997; Stamper and Tuovinen 1998; Corta et al. 1999). Degradation of chloroacetanilides and formamidines requires a consortium of bacteria for completion (De Schrijver and De Mot 1999). This mostly occurs in aerobic conditions and is modest under anaerobic conditions (Konopka 1994). Metolachlor is transformed to a lesser extent than alachlor, while amitraz is quickly transformed in the environment and in living organisms to the more acutely toxic intermediate BTS27271 before further degradation (FAO/WHO 1985; Knowles and Hamed 1989; Pass and Mogg 1991; Konopka 1994; Pass and Mogg 1995; Corta et al. 1999).

The fate of the parent compounds and their breakdown products are influenced by their solubility, stability, temperature, irradiation, biodegradability, bioavailability, runoff, precipitation, management practices, wind drift, erosion and chemical sorption (Becking et al. 1992; Allan 1994). High ambient temperatures, larger flux of the sun’s irradiation and torrential rains are characteristics of the tropics, which may enhance the rate of pesticide degradation and dissipation.

The occurrence of degradation products of chloroacetanilides in the tropics is not documented, while in the temperate zone the parent chloroacetanilide compounds and their degradation products have been found in surface water and groundwater (Galassi et al. 1996; Thurman et al. 1996; Albanis et al. 1998; Hostetler and Thurman 2000; Scribner et al. 2000b; Scribner et al. 2000a). Indeed, the potential risks of the pesticides in the environment should be a sum of the risks posed by the parent, intermediate, and stable degradation products. The analysis of the stable transformation products in water and the sediment is vital to evaluate the non-point-source contamination of water by chloroacetanilides and to estimate their long-term effects in the aquatic environment.
Fig 1: Designations and partial metabolic pathways of chloroacetanilide herbicides and formamidine insecticides showing possible routes resulting in conversion of pesticides to the mutagenic and carcinogenic nitrosobenzenes. Modified from Kimmel et al. (1986).
Test Organisms

Xenopus laevis larvae

The clawed frog *Xenopus laevis* is a native species in Sub-Saharan Africa. It is grouped in the family Pipidae, all of whose members are exclusively aquatic and tongue-less. They are easy to breed and raise in the laboratory. Furthermore, they allow induction of artificial spawning at any time of the year with the females laying 500 - 2400 eggs at each time (Dumpert and Zietz 1984). Human Chorionic Gonadotropin (HCG) hormone is used for breeding induction by injection into the dorsal lymph sac (Fig 2).

![Adult Xenopus laevis](image)

Fig 2. Adult *Xenopus laevis*. The triangle shows the position of the dorsal lymph sac

The amphibian embryo remains a classical model for experimental embryological studies as it is an intact developing system, which undergoes
evolutionary conserved events of cleavage, gastrulation, and organogenesis, comparable to those of other vertebrates, including mammals (Dumont et al. 1983b). Validation studies using compounds with known mammalian and human developmental toxicity, or both, suggest that the predictive accuracy of the *Xenopus laevis* embryo test approximates 85%, so it can be used as an indicator of potential human developmental health hazard (Dumont et al. 1983a; Courchesne and Bantle 1985; Sabourin et al. 1985; Dawson and Bantle 1987; Sabourin and Faulk 1987; Bantle 1995). The test with the *Xenopus* embryos is a standardized 96-h test using midblastula (stage 8) to gastrula (stage 9) stages, thereby exposing all the sensitive stages of primary organogenesis (Nieuwkoop and Faber 1975; ASTM 1991; Bantle 1995). An embryonic teratogenic index (TI; which is expressed as 96h-LC50/96h-EC50(malformation)) allows comparison of teratogenic risks of diverse compounds and mixtures (Dumont et al. 1983b; Bantle 1995). The teratogenicity of highly embryolethal compounds would obviously be less relevant in the environment compared to that of less lethal compounds, which have a potential to cause malformation in a large number of surviving organisms.

**Chironomus riparius larvae**

The dipteran family Chironomidae are sediment-inhabiting organisms with a cosmopolitan distribution in freshwater ecosystems (Armitage et al. 1995). Their first instar is planktonic, while the 2nd to 4th instar stages inhabit the upper layer of the sediment. The sediment is a major repository for many persistent chemicals, making *C. riparius* larvae ideal organisms to evaluate adverse effects of toxicants. At 20°C a complete life cycle of the *C. riparius* can be completed in three weeks, making the insect easy to culture in the laboratory. The 1st instar larvae have been chosen for the present study based on their relatively higher sensitivity than the later stages of development (Williams et al. 1986). The larvae were obtained from a laboratory culture at the Department of Aquatic Ecology and Ecotoxicology, which has been maintained at 20°C (16:7 h light-dark regime, separated by 0.5 h twilight). To minimize the risk of inbreeding, egg masses were regularly exchanged with other Dutch laboratory cultures of *C. riparius* and large insect populations were maintained.
General Introduction

Vibrio fischeri

*Vibrio fischeri* is a motile gram-negative bioluminescent marine bacterium with a rod shape. Luminescence in *V. fischeri* is initiated by a cell-density-dependent activation of the gene *lux* regulon- ‘Quorum Sensing’ (Dunlap and Kuo 1992). Each bacterium produces chemical signals called autoinducers, which upon attaining a threshold concentration induce the reactions leading to luminescence. A blue-green light at 480-490 nm is produced after a luciferase-catalyzed oxidation of flavin mononucleotide (FMNH$_2$) and a range of long chained (8 - 14C) fatty aldehydes (RCHO), the luciferins (Meighen 1993). Fig 3. summarizes the *Vibrio fischeri* light emitting reaction.

![LuxAB (Luciferase)](attachment)

Fig 3. The light emitting reaction of *V. fischeri*

Cytotoxic compounds or luciferase inhibitors will result in reduced luminescence, the endpoint in the acute toxicity test using *V. fischeri* (Microtox®). Microtox® is an inexpensive standardized short-term *in vitro* bioassay that can screen complex industrial effluents, environmental mixtures and newly introduced compounds.

Sublethal concentrations of genotoxic compounds, restore luminescence in a dark mutant (M169) of the *V. fischeri* in the Mutatox® test. Different genotoxic agents, including base substitution, frame shift, DNA synthesis inhibitors, DNA damaging agents, and DNA intercalating agents, result in the appearance of light in the dark strain of M169 (Ulitzur 1982). The multiple endpoint targets of the test system make it highly sensitive, hence suitable as a rapid and cheap screening assay for suspect genotoxicants.
Outline

The fate of massively applied chloroacetanilides, formamidines and their degradation products in the tropical environment is not known and it is suspected that this may be different from that in the widely studied temperate region. Therefore, river water and sediment samples from River Nzoia, Kenya, were analyzed for alachlor, metolachlor and their stable aniline degradation products (Chapter 2). An attempt was made to reconstruct their fate and to elucidate their potential risks. These risks were estimated by deducing the pesticide balance of the River Nzoia catchment. Based on the evidence that the pesticide degradation products are potentially more mutagenic than the parent compounds, our first set of laboratory experiments aimed to discriminate baseline toxicity from effects on specific biological endpoints. Toxicity of the pesticides, their degradation products and chemically related compounds were investigated using *Chironomus riparius* and *Vibrio fischeri* (Chapter 3). Genotoxicity of the compounds was explored using the MUTATOX® test. Potentially genotoxic compounds may affect especially developing embryos, given the inherent challenges of cell division, differentiation and rapid growth during this period. Therefore in Chapter 4 and 5 we examined the developmental and teratogenic effects of the commonly used chloroacetanilides (alachlor and metolachlor), formamidine (amitraz), their stable aniline degradation products and, additionally, the herbicide paraquat on early embryos of a native frog species, *Xenopus laevis*. The concluding remarks (Chapter 6) discuss the main findings of this thesis as well as the implication for risk assessment of pesticides in the tropics.
General Introduction

References


General Introduction


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