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Ecotoxicology and Genotoxicology
Non-traditional Terrestrial Models

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Preface

Many important ecosystems around the world are being constantly challenged owing to the growing human and industrial pressure exerted upon them. The use of various biomarkers in local, easily available species can be applied to evaluate the response of the biota to such pollutants. Several biological parameters mirror the interactions between toxic agents and biotic matrices. These are powerful tools that can be applied to environmental monitoring tests and studies. Their responses may reveal general deleterious effects to the organism, pinpointing alterations at a cellular, biochemical and molecular level, as well as higher levels of organisation.

Our global society needs to table down actions and set rules to evaluate and considerably reduce the real and potentially hazardous factors in the environment that can, as previously stated, result in health risks for all forms of life (including *Homo sapiens sapiens*). Despite major positive contributions in the field of health, owing to the immense progress achieved in science, technology and industrialization, the interaction between environmental risk and health is an often intricate equation, not self-evident, that involves a variety of not only social, political and economic, but also lifestyle factors. This cannot be emphasized enough. Health depends on the good quality of environmental ''basic ingredients'', such as air, water, soil and food, among others. We believe that the ultimate challenge in this matter is to weigh-in short-term positive gains, while, at the same time, taking into account long-term effects of substances used. Available information about the toxic effects of heterogeneous xenobiotics, continuously released into human habitats, inadvertently, deliberately, or by non-regulated industrial discharges on biological components of the environment, is inconclusive.

There is not a clear-cut definition of the concept of Environmental Health. Various openings help us in the understanding of this concept. According to

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the World Health Organization, it is defined by ''all the physical, chemical and biological factors external to a person and all the related factors impacting upon behaviours. It encompasses the assessment and control of those environmental factors that can potentially affect health. It is targeted towards preventing disease and creating health-supportive environments. . .'' For the National Environmental Health Association, this concept refers to ''the protection against environmental factors that may adversely impact human health or the ecological balances essential to long-term human health and environmental quality, whether in the natural or man-made environment.'' A third definition by the National Institute of Environmental Health Science also involves the criteria that ''the social environment encompasses lifestyle factors like diet and exercise, socioeconomic status, and other societal influences that may affect health.''

In general terms, our health and the health of many other species are negatively affected by five broad categories of environmental hazards, namely, electromagnetic fields (produced by high power lines, electrical wiring, appliances, mobile phones, computers, and TV sets, *etc.*), radiation (including nuclear fallout from weapons testing, fission materials from nuclear power plants and their respective accidents, leaking radioactive disposal sites, air travel and x-rays), toxic chemicals (some organochlorines, phthalates, polybrominated flame retardants, perfluorinated substances, bisphenol-A) and several toxic metals, among others, which have been shown to have endocrine-disrupting properties, and finally soil mineral depletion as a complex environmental hazard.

By definition, health risk assessment in its quantitative and/or qualitative determinations includes variants such as the type of risk involved and the severity of response, within or without a probabilistic context. In this regard, risk-based methods of analysis play a strategic role in identifying and ranking adverse responses or the structure of the effects of exposure *vis-à-vis* environmental factors.

Many compounds can be hazardous if not used appropriately and may present a real risk to the environment, contaminating soil, water and air. Most of the pollutants in the different environmental compartments exert their effects through cytotoxic, genotoxic and metabolically toxic mechanisms. In pollution studies, there is an increasing interest in biomonitoring markers of biological exposure to pollutants. To achieve this goal, several end-points for the three above-mentioned factors have been used in aquatic and terrestrial invertebrate and vertebrate species on contaminated areas (*in situ* assays) and to screen for xenobiotics after direct or indirect exposure (*in vivo* assays).

The use of invertebrate and vertebrate autochthonous species as indicators for monitoring pollutant-induced deleterious environmental effects will raise the current awareness of real and potential hazards. It is also known that most of the environmental pollutants not only affect target organisms, but concomitantly exert negative effects on non-target species as well.

Invertebrate and vertebrate animal models have been used for decades in acute and chronic toxicity tests for hazard identification. They can be very efficient screening systems that have a major role to play in toxicity research, because certain aspects of their biology, physiology and genetic characteristics make them suitable models in ecotoxicological and genotoxicological studies.

These two books intend to provide an overview of the use of non-conventional, locally available, invertebrate and vertebrate species as experimental models for the study of different toxicological aspects induced by environmental pollutants in both aquatic and terrestrial ecosystems. Volume One, *Ecotoxicology and Genotoxicology: Non-traditional Aquatic Models* includes examples of the use of aquatic species or aquatic stages of terrestrial species and Volume Two, *Ecotoxicology and Genotoxicology: Non-traditional Terrestrial Models,* is committed to terrestrial non-conventional animal models.

Both volumes aim to shed some light on the matter, whilst offering relevant tools for evaluating risk and to provide a framework for practical discussions. These will foster decisions and actions required to reduce environmental health risk against environmental factors. This piece of work has been systematized for the sake of clarity, presenting some real-life examples and extending concepts (of hazardous factors) to living species that may stimulate new research ideas and trends in the relevant fields.

Available information has been compiled from a diversity of sources, trying to achieve a representative global and geographical balance, as far as possible, whilst at the same time aiming at high-quality studies. We believe that this piece of work is unique in this sense.

Many researchers from different parts of the world have contributed to the publication of this book. Given the fast pace of new scientific publications shedding more light on the matter, these books will probably be outdated very soon. We regard this as a positive and healthy fact. We hope that these books will meet the expectations and needs of all those interested in the environmental risk assessment field of study by the use of widely available species worldwide. Finally, we also hope that the examples included in the different chapters of these books will awaken the ability to search for new organisms in local and regional ecosystems to pursue further studies in ecotoxicology and genotoxicology. If our wishes are granted, we shall be happy to oblige and edit the next edition of this series.

Prof. Dr Marcelo L. Larramendy
and Dr Guillermo Eli Liwszyc

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Section I: Terrestrial Invertebrates as Experimental Models

CHAPTER 1

The Use of Non-standardized Invertebrates in Soil Ecotoxicology

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1.1 Soil Invertebrates

From the approximately 8.7 million species estimated to be living on planet earth, about 7.7 million are represented by individuals of the animal kingdom,[1] and only about 5% of these animals are represented by those who have a backbone, known as vertebrates. All the others, representing the major part of the animal kingdom, are known as invertebrates.[2] In general, invertebrates are multicellular animals that do not have and do not develop a vertebral column derived from the notochord.

All existing invertebrates are distributed through about 35 phyla, but the number of phyla may vary according to the chosen classification. Most of

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them belong to aquatic environments (especially the marine one), although there is a considerable percentage that inhabit terrestrial ecosystems.[2] It is estimated that the animal species living in the soil (including those that spend at least a part of their lives in soil) represent 23% of the total described species of the terrestrial environment (considering all kingdoms). However, more than 22% are represented exclusively by invertebrates,[3] which shows that soils comprise a high diversity of invertebrate species.

Although the diversity of species in the soil can be quite variable, when considering the different types of soil and climate conditions, the majority of the species of soil invertebrates are distributed among the phyla Arthropoda, Mollusca, Porifera, Cnidaria, Platyhelminthes, Nematoda, Annelida and Echinodermata.[2] However, approximately 85% of all of these invertebrates belong to the Arthropoda phylum. Therefore, to make a weighted representation of the invertebrate diversity in soils, this should mainly consist of Insecta, Arachnida, Mollusca, Diplopoda, Annelida and Nematoda,[2] with the highest species richness in those taxonomic groups.[2]

Because it is the easier procedure, for a long time classification of soil animals has been based on body size (length and width).[4] According to several authors,[5,6] the following groups were established as a result of this classification: soil microfauna (less than 0.1 mm), mesofauna (0.1 to 2 mm), macrofauna (2 to 20 mm) and megafauna (larger than 20 mm). The majority of the soil invertebrates can be found in the groups of micro-, meso- and macro-fauna, while megafauna is only represented by insectivorous vertebrates, rodents, as well as some larger sized invertebrates.[2]

This classification includes animals with life cycles ranging from a few days to more than 10 years and it is known that the smaller (micro- and meso-fauna) or the larger organisms (macro- and mega-fauna) are generally related to different specific functions in the soils.[5,7] Therefore, the role of the microfauna is to act as controllers of the microbial soil populations, while the role of the macrofauna is the fragmentation and distribution of the organic material along the soil profile. However, it is important to understand that there is no direct correlation between the size of a soil animal and its trophic position in the food chain.[2]

Soil microfauna is composed of microscopic animals (<0.1 mm), which are the most abundant invertebrates on earth. This classification of small-sized animals encompasses protozoans (Protozoa), nematodes (Nematoda), rotifers (Rotifera) and tardigrades (Tardigrade), among others, which normally inhabit the water film in the soil pores. In general, these organisms have short life cycles and belong to different functional groups, which are classified in accordance with their feeding preferences, such as those that feed essentially on other animals (parasites/predators), on plant roots (phytophagous) or on micro-organisms (*e.g.* fungivorous and bacterivorous). In this sense, during their activities the microfauna stimulate nutrient mineralization as well as the control of microbial populations in the soil.[7,8]

The soil mesofauna comprises invertebrates with body diameter (in width) between 0.1 and 2 mm.[5] In this taxonomic class of animals we find small

enchytraeids (Enchytraeidae), pseudo-scorpions (Pseudoscorpionidae), mites (Acari), springtails (Collembola), Diplura, Protura, symphylans (Symphyla), pauropodas (Myriapoda), insects (*e.g.* micro beetles and ants), spiders (*e.g.* small individuals of the family Araneida), and other small arthropods.[7] In general, the predominant eating habit of the mesofauna is detritivorous (feed on litter), fungivorous and/or predators (feed on fungal hyphae and/or on individuals of microfauna, especially nematodes and protozoa). When feeding on litter, these invertebrates expand the contact surface of the organic materials, favoring the microorganism's attack and, consequently, improving the decomposition rates and the nutrient mineralization that favor the plants. Therefore, although the individuals of the mesofauna do not strongly contribute to the overall soil biomass and respiration, they play a key role as regulators of decomposition processes.[9]

Macrofauna encompasses more than 20 taxonomic groups with a body size between 2 and 20 mm,[5] as described below. They are earthworms (Oligochaeta), termites (Isopotera), ants (Formicidae), centipedes (Chilopoda), millipedes (Diplopoda), cockroaches (Blattodea), spiders (Arachnida), earwigs (Dermaptera), crickets (Orthoptera), snails (Gastropoda), scorpions (Scorpiones), stink bugs (Hemiptera), cicadas (Cicadoidea), woodlice (Isopoda), moths (Thysanura), flies (Diptera) and butterflies (Lepidoptera) larvae, and adult beetles (Coleoptera), among others.[2,5,7]

The soil macrofauna eating habits can be quite varied.[7] They can be soil consumers (geophagous), or feed on living parts of plants (phytophagous) including roots (rhizophagus), or on soil organic matter (humivorous), on litter (detritivorous), on wood (xylophagous), on other animals (predators, parasites or necrophagous) or on fungal hyphae (fungivorous). Regardless of their feeding habits, soil macrofauna perform a key role in fragmenting and transporting the organic material (vegetable and animal debris) through the soil profile. In addition, during their transportation activities in the soil profile, most of them (especially termites, carabid beetles, ants, millipedes and earthworms) create biogenic structures (galleries, nests, chambers or fecal pellets), which may modify the physical properties of soils, as well as change the availability of food resources for other organisms. Therefore, they are also called ''Ecosystem Engineers''.[7,8,10]

Megafauna consist of animals with a body width bigger than 20 mm and are almost entirely vertebrate animals. Therefore, sometimes they are called ''Soil Vertebrates''.[5] Among the representative species of the megafauna are small mammals and some rodents, amphibians and reptiles, in addition to a few larger invertebrates, normally represented by giant earthworms.[5] Though some larger mammals (*e.g.* hares, rabbits, hedgehogs and foxes) can build their burrows in the soil, they are not considered part of the soil megafauna.[5] With the exception of earthworms, which are geophagous, the megafauna has predominantly a predatory alimentary habit, feeding on smaller invertebrates (macro- and meso-fauna) and on parts of living plants (*e.g.* leaves, stems, roots, seeds). During their activity, the megafauna create great galleries and structures in the soil profile, though in much smaller numbers than the macrofauna.

1.2 The Use of Invertebrates in Soil Ecotoxicology

As already shown in the previous section of this chapter, unlike plants that produce their own energy through photosynthesis, soil invertebrates need to extract the energy needed for survival from other living organisms. This is why they feed on a great diversity of autotrophic, as plants, heterotrophic, as animals, and microorganisms, and occupy several positions in the food web. This fact, added to other varying behavioral habits of invertebrates in the soil, may result in significant differences for the maintenance of terrestrial ecosystems.

The changes promoted by soil organisms, especially those that benefit human beings and the environment, are known as ''Ecosystem Services''.[11] The importance of the services provided by soil invertebrates in terrestrial ecosystems has been extensively discussed in the literature over the last decade,[3,7,11,12] especially of those who benefit the agricultural ecosystems (agriculture and forestry).

Anthropogenic activities often produce negative impacts on the environment, for example, the impacts of conventional agricultural practices, such as pesticide application, use of chemical fertilizers and waste disposal, among others. They cause stresses on the living organisms of the terrestrial ecosystems and produce negative effects on some of the essential soil ecosystem services.[11] For this reason, researchers in the areas of soil health and soil quality have been warning about the imminent need for developing effective techniques to identify, quantify and prevent the impacts of toxic substances or elements on soil invertebrate species and thus establish limits to protect their associated ecosystem services.

Nevertheless, only in recent years have the evaluations of the ecological risk of contaminants on soil health started to use living organisms as indicators (bio-indicators), as substitutes or complementary to the traditional chemical and physical analyses.[11] For a long time, scientists believed that the biological properties of soils were more difficult to predict or even to measure.[13] However, bio-indicators can be practical tools in this type of assessment because they are highly reactive to the environmental conditions.[14] Changes caused by toxic substances in soil are perceived quickly by analyzing the changes of the living soil community in their presence. Diversity, distribution and vital functions of soil inhabitants as well as the soil's chemical composition allow us to draw conclusions regarding the quality of contaminated soils. Therefore, nowadays, there are in use numerous protocols of studies using species and communities of soil invertebrates as biological indicators of the impacts of the contaminants in terrestrial ecosystems.

A recent study describes ecotoxicology as a scientific area that studies the effects of chemicals on living organisms in the environment with the final goal of protecting the structure and function of the ecosystems.[15] Thus, methods using soil invertebrates to assess the toxicity of substances disposed on soils are known as Soil Ecotoxicological Assays. This type of testing

is based on the principle that the exposure of living organisms to stressful factors, for example those generated by toxic substances, has a particular tendency to change some vital functions (at different levels), allowing us to measure the toxicity of the substances on species, populations and even communities.

Ecotoxicological tests with soil invertebrates can be applicable to many classes of contaminants, may be performed in a short time (for example, in 48 hours), and may be a low-cost option. These tests allow for assessment of a wide range of toxicity types and biochemical, physiological, morphological and behavioral endpoints on organisms.

They have been used to detect the presence, concentration and mode of action of a soil contaminant on the soil organisms and to identify changes and early signs of pollution in the terrestrial environment. Furthermore, they have been used to determine cause–effect and dose–response relations between toxic agents and selected species, to complement the chemical and physical soil analyses in order to indicate the quality of terrestrial ecosystems; and finally, to evaluate the effectiveness of bioremediation management in polluted soils.[16,17]

In general, the aim of soil ecotoxicological assays can be achieved by assessing the toxic effects of chemicals/elements by using single-species tests, under controlled laboratory conditions. Based on the effect concentrations obtained in these tests, it is possible to establish safe limits of exposure for populations and communities living in natural environments. Especially in the case of the prospective risk assessment of chemicals, laboratory tests show a clearer causal relationship between exposure and effects than field assays.[18]

In the case of laboratory tests, the key measuring parameters on invertebrates are related to survival (the number of living/dead individuals), growth (the body biomass or animal body length), reproductive success (the ability to generate viable individuals) and behavioral disorders (as walking ability and avoidance behavior) owing to exposure to contaminants.[11] There are studies based on more complex methodologies on micro- and mesocosm levels, or at field levels, in order to reduce the uncertainty about the toxic risk on invertebrate soil species.[17] In this type of assay, it is possible to evaluate changes in the activity, abundance and diversity of natural soil invertebrate communities from terrestrial ecosystems, and to also assess the direct impact of pollutants on fundamental ecosystem services of human interest. One example is the use of litter-bags to evaluate the consumption of plant material deposited on the soil by the invertebrate fauna in contaminated sites.[19] However, such methods may be expensive, require more labor, are time-consuming and need an integrated analysis of the risk factors.

Although the use of invertebrates to assess the toxicity of substances in soil has been reported since the 1960s,[15,20,21] it was only about 30 years ago that the first standard protocols describing methodologies for laboratory toxicity tests with soil invertebrates were published.[11] According to a literature review of the history of soil ecotoxicology, these international protocols have emerged to support the risk assessment procedures of chemicals in soil

as well as to assist in pesticide registration in some countries; consequently they got greater attention of investigators in the related study areas.[15]

Currently, soil ecotoxicology is booming, a fact that can be confirmed when looking at the number of published articles indexed in the database "ISI Web of Science" under the terms "Soil" + "Ecotoxicology" over the few past years. In 2015 this number was about 30 times higher than that of publications in 1992. According to recent literature reviews on this subject, this increase in scientific production is related, among other factors, to a great development in soil ecotoxicology in recent years, highlighted both by the new lines of research in the area and by improvements on traditional laboratory toxicity tests.[11,15,22,23]

Among the new lines of research and of improvements in traditional techniques we include: changes in the constituents of artificial soil[24,25] proposed by the OECD,[26] as well as the replacement of artificial soils (original or modified versions) by natural soils, for example, LUFA soils,[27] and changes in the laboratory climatic conditions (*e.g.* temperature) for species cultures and assays, in order to increase the realism of studies performed under tropical climatic conditions,[24,28,29] and the development of new types of toxicity tests, in order to observe different endpoints (*e.g.* avoidance behavior assays with earthworms and springtails[30,31] are included). Evaluation of the toxicity of mixtures of contaminants and the assessment of the influence of climate changes on the toxic potential of substances against soil invertebrates[32,33] are also included. Finally, with even greater importance for this chapter, research looking for new alternative species of soil invertebrates for the standard laboratory toxicity assays,[34–39] or those with the unique aim of increasing the ecological relevance of non-standardized assays carried out to obtain more accurate responses about specific local ecosystems, play increasingly important roles.

The selection of new test species is particularly pertinent for soil ecotoxicology when taking into account the consistent demand for increasing the representation of the existing taxonomic groups of soil invertebrates in toxicity testing. According to some authors,[12,17] in an ideal situation, the toxicity of all substances deposited on soils should be measured on all animal species from a particular ecosystem, prior to establishing a limit of generic exposure to preserve the invertebrate's biodiversity. However, these authors themselves recognize that this is an impossible task to fulfill through laboratory toxicity tests, if one considers that the diversity of species in soil macro-, meso- and micro-fauna is in the order of magnitude of millions, as described in the first section of this chapter.

Despite the large numbers of taxonomic groups available to be used as bio-indicators for toxic effects of substances in soil,[15,35] only a few species of earthworms, enchytraeids, mites, collembolans, nematodes, mollusks and insects of the family Carabidae were selected for the soil ecotoxicological assays standardized by the main international regulatory agencies (Table 1.1). Those agencies include the American Society for Testing and Materials (ASTM), Environment Canada, the International Organization for

Table 1.1 Summary of the available standard invertebrate species for soil ecotoxicological assays. Table derived from C. A. M. Van Gestel, Soil ecotoxicology: State of the art and future directions, *ZooKeys*, 2012, **176**, 275–296. Copyright 2012 C. A. M. Van Gestel. This was published under the Creative Commons Attribution License 3.0 (CC-BY) (https://creativecommons.org/licenses/by/3.0/legalcode).

Group	Test organism	Species	Endpoint	Test type	Guideline/Reference
Oligoachaetes	Earthworms	*Eisenia andrei*	Survival; growth	Lab. toxicity test	OECD 207;[26] ISO 11268-1;[40] EPA 712-C-016;[41] EPS 1/RM/43[42]
		Eisenia fetida	Reproduction; growth	Lab. toxicity test	ISO 11268-2;[24] EPS 1/RM/43;[42] OECD 222[43]
		Lumbricus terrestris	Avoidance	Lab. behaviour test	ISO 17512-1;[30] EPS 1/RM/43[42]
			Bioaccumulation	Lab. toxicity test	ASTM E1676-12[44]
		Different species	Species diversity and abundance	Field test	ISO 11268-3[45]
	Enchytraeids	*Enchytraeus albidus*	Survival; reproduction	Lab. toxicity test	OECD 220;[46] ISO 16387[47]
		Other *Enchytraeus* spp.	Bioaccumulation	Lab. toxicity test	ASTM E1676-12[44]
Arthropods	Collembolans	*Folsomia candida* *Folsomia fimetaria*	Survival; reproduction	Lab. toxicity test	EPS 1/RM/47;[48] OECD 232;[49] ISO 11267[50]
		Orthonychiurus folsomi *Proisotoma minuta*	Avoidance	Lab. behaviour test	ISO 17512-2[31]
	Mites	*Hypoaspis aculeifer*	Survival; reproduction	Lab. toxicity test	OECD 226[51]
	Carabid insects	*Oxythyrea funesta*	Survival	Lab. toxicity test	ISO 20963[52]
Nematodes	Nematodes	*Caenorhabditis elegans*	Survival	Lab. toxicity test	ASTM E2172-01[53]
Mollusks	Snails	*Helix aspersa*	Survival; growth	Lab. toxicity test	ISO 15952[54]

Standardization (ISO), the Organisation for Economic Co-operation and Development (OECD) and the US Environmental Protection Agency (EPA).

The current number of available species for the standard laboratory ecotoxicological assays (Table 1.1) is considered too low, and demonstrates an under-representation of the diversity of invertebrates inhabiting soils in natural ecosystems or even when compared to the diversity of invertebrate fauna in soils from the agro-ecosystems. The sub-representation is even greater when comparing the number of arthropods with the number of earthworm species selected for the standard tests.[15] The species richness of earthworms in terrestrial ecosystems is generally much smaller than the richness of arthropod species, but these oligochaetes are represented by at least five test species in standard toxicity assays (*Eisenia andrei*, *Eisenia fetida*, *Lumbricus terrestris*, *Enchytraeus albidus*, *Enchytraeus crypticus*—Table 1.1), in addition to a field test with different earthworm species.[45] On the other hand, arthropods comprise about 80% of all soil invertebrate animals and are only represented by the springtail species *Folsomia candida*, *Folsomia fimetaria*, *Orthonychiurus folsomi* and *Proisotoma minuta*, and by the species *Hypoaspis aculeifer* (predatory mites) and *Oxythyrea funesta* (Carabid insect).

Within the limited number of species available for standardized toxicity tests (Table 1.1), only *E. andrei*, *E. fetida*, *F. candida*, *E. albidus* and *H. aculeifer* (Figure 1.1) have been routinely used in batteries of ecotoxicological assays to assess the ecological risk of substances for the soil fauna. It is also possible to conclude that the representativeness of soil invertebrates is further reduced in a higher degree than is supposed by the recent reviews on this subject.[11,15,17,22] According to a number of studies,[55–58] this poor representation of the soil fauna in ecotoxicological testing is, in great part, a consequence of the stringent requirements for the standardization of the assays, which, in general, choose the species based on rigid parameters, such as:

(a) Physiological: the species must have high sensitivity to soil contaminants, low variability (use preferably species with parthenogenetic

Figure 1.1 Summary of the available standard invertebrate species for soil ecotoxicological assays: (A) *Eisenia andrei*; (B) *Folsomia candida*; (C) *Enchytraeus crypticus*; (D) *Hypoaspis aculeifer*.
Photos reproduced with permission from: (B) C. M. Ribeiro, picture of the species *Folsomia candida*; (A) and (C) C. A. Santos, pictures of the species *Eisenia andre* and *Enchytraeus crypticus*; (D) M. Bianchi, picture of the species *Hypoaspis aculeifer*.

reproduction), high reproduction rates, short generation time, and be tolerant to several culturing substrates and artificial soils.

(b) Functional: they must be abundant and representative of a taxonomic group and, preferably, should belong to functional groups with essential services for the functioning of the terrestrial ecosystems.

(c) Practical: species with well-known biology, easy to identify (by taxonomy based on morphology), cultivate, maintain and synchronize (age and/or size) under laboratory conditions. It is also desirable to choose species with a reasonable number of measurable responses (*e.g.* pollutant concentration in tissues, biological disorders in growth and fertility, or genetic changes).

Methods based on standard invertebrate species, described in the International Standards, as those published by ASTM, Environment Canada, ISO, OECD and EPA (Table 1.1), are essentially designed to simplify the comparison between results of different laboratories (regardless of geographic location), as well as to increase the accuracy of the dose–response relationships obtained between substances and test species. This accuracy is fundamental in order to establish appropriate protective limits of exposure for invertebrates in polluted soils, especially in assays performed to release new molecules (*e.g.* of pesticides) to the market. On the other hand, it should also be admitted that the dependence on results only based on standard species may underestimate the real impact of toxic substances in terrestrial ecosystems, since it is not possible to prevent the impacts (*e.g.* species extinction or the loss of ecosystem services) on the immense diversity of invertebrates existing in the soil.[3,8]

For this reason, it is necessary to continue further studies in order to select standard species for toxicity testing, especially among soil arthropods, to improve the representativeness of these organisms in the ecological risk assessments of pollutants. In this sense, species of the order Isopoda (*Porcellio scaber*, *Oniscus asellus* and *Porcellionides pruinosis*) are strongly recommended for inclusion in standardized ecotoxicological assays.[15] Isopoda species have great potential for standard assays because of their high ecological significance in terrestrial ecosystems. Thus, their typical routes of exposure to soil pollutants (by contact and ingestion) and the interesting characteristics of their life cycle, with endpoints alternative to the traditional ones, are promising. In addition, they have been used in toxicity tests for more than 30 years.[15]

Other activities are also being proposed for the selection of new species in soil toxicity tests. Some authors proposed that enchytraeid species of the *Fridericia* genus (*Fridericia bulbousa* and *Fridericia peregrinabunda*) should be considered as new test species in the list of standardized assays for soil ecotoxicology.[37,39] According to these authors, those species have similar (or higher) sensitivity to some heavy metals and pesticides, when compared to some of the standard soil invertebrates. In addition, the *Fridericia* genus has

greater representation in terrestrial ecosystems, because this is the genus in the Enchytraeidae family with the greatest species richness of worldwide distribution.[59]

On the other hand, the possibility of using toxicity assays with non-standard (alternative soil invertebrate) species cannot be dismissed. During the last few decades, following the development of standardized toxicity tests, there was also an increase in the number of ecotoxicological studies using non-standard invertebrate species.[15] In general, the research using alternative species has similar objectives to the standardized assays. These types of assays assess the same endpoints as the standardized assays (*i.e.* survival, reproduction and behavior), however, in view of the characteristics of the selected species.

An advantage of the use of alternative species in terrestrial ecotoxicological tests is the higher representation of the local diversity of soil invertebrates. Using species from a particular biome will help to increase the accuracy of the responses for the local fauna, when simulating the impacts in a specific natural ecosystem. Springtails, such as *Onychiurus armatus*, *Protaphorura quadriocellata*, *Orchesella cincta*, *Tullbergia granulata*,[60] *Proisotoma minuta*[61] and *Isotoma* viridis,[62] are used as bio-indicators of soil pollution and are examples of alternative species to increase the ecological relevance of risk analyses of substances in terrestrial ecosystems. The main standard species of springtail (*F. candida*) is recommended in standard assays,[31,42,49,50,55] but has limited ecological relevance, because of its absence in many natural and agricultural habitats.[63]

Moreover, studies based on alternative species have not received the proper attention for several reasons, such as those enumerated below:

I. Many of the selected alternative species are still unknown for most of the researchers working with the soil matrix and, therefore, their use is unusual.
II. These species are native to specific biomes and are not easily found (and identified) in places in which most of the researchers are interested in (they have low ecological relevance in other ecosystems, different from those of origin).
III. The organisms do not have a well-known biology (or it is even unknown) and/or are not suitable for the traditional methods of cultivation under laboratory conditions.
IV. Some species have very long life cycles, which makes it difficult to assess certain parameters, such as reproduction, effects on longevity and heritable genetic damage.

The next section of this chapter will show examples of the use of alternative species in soil ecotoxicology. Only Earthworms, Collembolans, Enchytraeids, Isopods and a few other taxonomic groups will be considered in the following discussion because of their recognized potential for ecotoxicological assays.[12,14]

1.3 Key Groups of Invertebrates for Soil Ecotoxicological Testing

1.3.1 Earthworms

Earthworms are the most frequently used organisms in standardized soil ecotoxicity tests around the world, and even among non-standard species they are the most used. The reasons are related to their ecological importance and the ease of conducting these tests. Earthworms are considered ecosystem engineers in soil because of their key role in soil structure and biological activity, *e.g.*, by building biopores, transferring organic material from the surface to deeper layers, producing humus, improving microflora, micro and mesofauna activity, and increasing plant growth.[8,64,65]

One of the weaknesses of using *Eisenia* spp. (species recommended by standardized guidelines) is the fact that *Eisenia andrei* and *Eisenia fetida* are epigeic species (litter dwelling), *i.e.*, they live and feed only on the surface. Such ecological traits may not represent what happens to other groups of Oligochaetes in soils, *e.g.*, the anecic and endogeic species, which are soil dwellers.[66] Other questions are with regard to their sensitivity to contaminants and representativeness of exposure conditions to different soils and climatic scenarios.[25]

Efforts have been made to identify potentially useful species for different ecozones,[25,67,68] *e.g.*, the earthworm species *Lumbricus rubellus*, *Dendrobaena octaedra* and *Dendrodrilus rubidus* were selected as the most promising candidates for Canadian boreal forest ecosystems.[69] In subantarctic conditions, the toxicity of diesel-contaminated soils was evaluated using the subantarctic earthworm *Microscolex macquariensis* in lethality, avoidance and 2 week reproduction tests. These tests were carried out at 8 °C, a realistic condition for the region. The species reproduced in laboratory tests, but the authors reinforced the need for more studies about its life cycle.[70]

Among the non-standard species studied in ecotoxicity tests, *Pontoscolex corethrurus* Müller (Figure 1.2A) is an endogeic species that shows a wide tolerance to environmental variations, living in many different habitats and soil types throughout the tropics and sub-tropics. Ecotoxicity tests were carried out using the existing guidelines for avoidance behavior and lethality tests, and this species showed similar sensitivity to the standard species *E. andrei* to the pesticides carbendazim, carbofuran and glyphosate.[71] These results could indicate that *E. andrei* is sensitive enough to represent the populations of this autochthonous species. However, this should not be generalized, considering other non-standard species or even other contaminants. *P. corethrurus* was more sensitive to the fungicide carbendazim, but less sensitive to the insecticide lambda-cyhalothrin, when compared with the standard test species *E. fetida* in lethality tests under tropical conditions.[25] *Metaphire posthuma* is an Indian species widely distributed in various states of India and other Asian countries that is well adapted to burrowing. This species was more sensitive to the pesticides carbaryl,

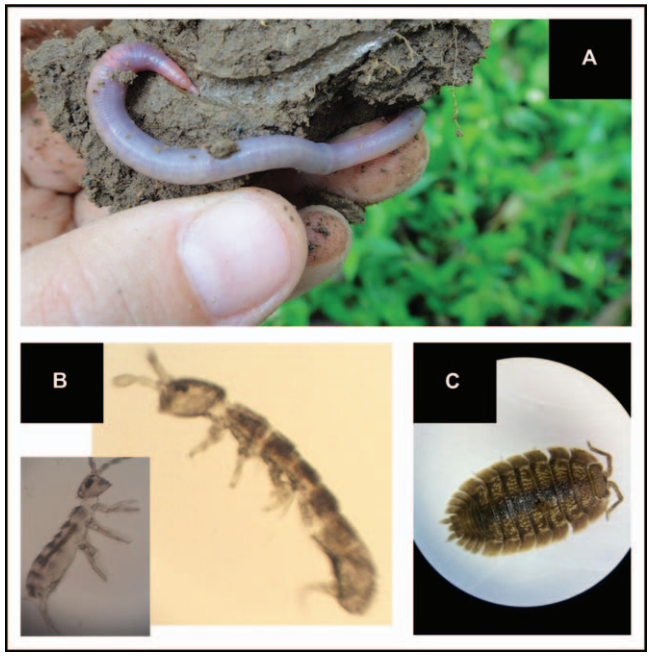

Figure 1.2 Examples of alternative species of soil invertebrates for the standard laboratory toxicity assays: (A) *Pontoscolex corethrurus*; (B) *Proisotoma minuta*; (C) *Porcellio dilatatus*.
Photos reproduced with permission from: (A) M. L. C. Bartz, picture of the species *Pontoscolex corethrurus*; (B) A. Buch picture of the species *Proisotoma minuta*; (C) L. P. Crescencio, picture of the species *Porcellio dilatatus*.

carbofuran, cypermethrin and fenvalerate than *E. andrei* in lethality tests.[72] *Octolasion tyrtaeum*, a common species in many Argentine agricultural ecosystems, was more sensitive than *E. andrei* to glyphosate, when testing adult lethality and variation of biomass.[73] Accumulation and toxicity of metals (Cu, Cd, Ni, and Zn) were studied using *Lumbricus rubellus* (epigeic), *Aporrectodea longa* (anecic), and *Eisenia fetida* (ultra-epigeic) with 28 days of exposure in two soils; under these circumstances, *L. rubellus* was the most sensitive species.[66] In ecotoxicological assessment of imidacloprid, *E. fetida* responded with significant avoidance behavior in all tested concentrations, while *L. terrestris* and *A. caliginosa* did not avoid the contaminated soil.[74] Differences in sensitivity or in bioaccumulation rates among species can be related to differences in ecological strategies (and, consequently, differences in exposure); to physiological mechanisms of detoxification and elimination;[66,75] or to differences in average body mass and surface/volume ratio.[74] Even a hormesis effect was detected in *Lampitto mauritii* for low concentrations of methyl parathion.[76] In general, uncertainty remains regarding the sensitivities of non-standard species in comparison to standard species.

The main challenges to using native earthworm species are the rearing of these organisms in the laboratory.[73] This is one of the reasons why reproduction tests were rarely conducted with non-standard species. The published works with native species reported field collection and laboratory acclimatization of earthworms before the tests, or reported buying earthworms in fishery stores. However, laboratory cultures are important to ensure the quality of test organisms, allowing synchronization of the cultures and avoiding exposure to contamination sources and abiotic stress.[77] Furthermore, field collections can be limited by seasonality in earthworm abundance or risks of confounding species. The difficulty is that some species have a long life cycle and low reproduction rates in the laboratory, *e.g. P. corethrurus*, whose life cycle was completed in about one year in laboratory studies.[78]

Still another consideration is that laboratory studies with non-standard species require knowledge of the biology and ecology of these species, once laboratory cultures and tests must be kept at optimal levels to avoid the influence of such interferences on the evaluated endpoints.[77] Adaptations of the existing guidelines have been made mainly regarding the duration of the reproduction tests, temperature, and amount of soil per replicate, in accordance with the ecological characteristics of the species.[25,70,76] Among the critical factors are temperature, pH, humidity and food quality. Cattle and horse manure are frequently recommended and have been successful for laboratory cultures. Other food sources cited are fresh leaves and litter, depending on the ecological characteristics of each species. The best culture medium for the epigeic species *Amynthas cortices* Kinberg includes sawdust, and this may be related to the presence of certain enzymes that help this species to degrade cellulosic compounds;[79] it was recommended to add mixtures of high and low quality organic residues in order to maintain successful field populations of endogeic and epiendogeic species in laboratory. Much work still must be done to increase the knowledge on the biology and ecology of the native species, their niches and needs, making it possible to select a larger set of species for the ecotoxicity tests.[80]

A new simple behavior test was proposed using *Lumbricus terrestris*, a common epi-anecic species, based on earthworm bioturbation and cast production.[81] Cast production was influenced by several factors, such as pesticide contamination[81] and soil quality.[82] Cast production rates were evaluated with *P. corethrurus* exposed to metal-contaminated soils and it was found to be a sensitive endpoint.[83]

Another interesting contribution to the test array is an alternative arrangement of avoidance tests, comprising two layers of soil, where the vertical burrowing behavior of *Allolobophora chlorotica* was studied when carbendazim contaminated soil was added to the surface of an unamended soil, containing the earthworms, and when the earthworms were laid on the surface of contaminated soil.[84] Earthworms significantly altered their burrowing behaviour to avoid carbendazim, but when they were added to an

upper layer of carbendazim-contaminated soil, they remained in this layer, probably because of neuronal impairment.[85]

In general, the studies reinforce the importance of using a multiple selection of species in ecotoxicology, considering different ecological strategies and including relevant species, aiming at predicting harmful environmental effects more accurately. Furthermore, considering the ecological requirements of important terrestrial ecotoxicological test species, most of the standard species are applicable to a wide range of natural soils, while for some ''extreme'' soils (*e.g.*, very acidic forest soils) alternative test species will be required.[58,68]

1.3.2 Collembolans

Collembolans are active and abundant under most environmental conditions and their activities usually mobilise C and N.[86] They are involved in complex trophic relationships in soil and are an important prey group for generalist arthropod predators in agro-ecosystems.[87,88] In opposition to the recognition of the potential value of this group as bio-indicators of soil quality, basic information about the occurrence and ecology of species is still largely unknown.[89]

Defining food preferences and optimal abiotic conditions is important to guarantee successful breeding in the laboratory. In a food choice test performed with the non-standard collembolan species *Protaphorura fimata* and *Heteromurus nitidus* with baker's yeast, *Saccharomyces cerevisiae*, and unicellular green algae, *Pseudokirchneriella subcapitata*, as food choices, both species preferred yeast.[88] However, different species can feed on fungi, bacteria, protozoa, algae, enchytraeids, nematodes, invertebrate remains and plant tissue.[86,90]

Temperature, moisture and pH seem to be the most important abiotic factors. Besides that, biotic interactions play a key role in the environment, especially related to microbial activity and competition with other faunal species. In laboratory cultures, contamination with predatory mites is a common problem and should be avoided by checking the food quality and the cleaning of the culture environment (recipients, room, and entomological aspirator).

The OECD guidelines[49] present a list of alternative species to be used in reproduction tests, when some prerequisites are attended to, such as: *Proisotoma minuta* (Figure 1.2B), *Isotoma viridis*, *Isotoma anglicana*, *Orchesella cincta*, *Sinella curviseta*, *Paronychiurus kimi*, *Orthonychiurus folsomi*, *Mesaphorura macrochaeta*. Among the prerequisites are: the unequivocal taxonomic identification, knowledge about the life cycles of organisms before the test, and optimal conditions for the species.

Non-standard species have been collected from a range of terrestrial habitats using a variety of techniques, such as suction sampling from vegetation, pitfall traps, and extraction from soils by flotation on water or by the use of Tullgren funnels. Usually the species are cultivated on the same

mixture of Plaster of Paris and powdered activated charcoal recommended by the guidelines for *F. candida*.[91]

However, establishment of laboratory cultures of Collembola collected from the field can be a difficult task. Some species of Australian Collembola were collected but did not produce viable cultures in the laboratory.[61] *Sminthurides* sp. and *Entomobrya* sp. collected from forest soils in Brazil showed low adaptation to laboratory conditions.[91] Species with characteristics such as small size, low reproduction rates or more conspicuous colour can be a challenge for use in ecotoxicity tests. *Sinella communis* was shown to be a suitable species for toxicological testing in Australia, being easy to count and being more sensitive to a range of toxicants than *F. candida*.[61] *Folsomia nivalis* was used in a battery to assess boreal forest soils,[68] and was found to be more sensitive than the standard species *F. candida* to hydrocarbon-impacted soils.[69]

Laboratory avoidance tests were conducted with five collembolan species (*Isotoma anglicana*, *Heteromurus nitidus*, *Lepidocyrtus violaceus*, *Folsomia candida*, *Onychiurus armatus*) towards the herbicide Betanal (active ingredient: phenmedipham) in soil.[92] Sensitivity was dose-dependent and species-specific, with *O. armatus* being the most sensitive species. At higher concentrations (near the calculated LC_{50} value), however, a higher number of organisms were found in contaminated soil, which can be a possible narcotic effect of this substance.

Soil characteristics can alter the toxicity of contaminants and also act as stressors themselves, as observed during the reproduction of *Paronychiurus kimi* in cadmium-contaminated artificial soil.[93] However, papers are scarce about the sensitivity of non-standard species to contaminants and about the influence of soil properties.

In conclusion, many advances are needed to increase the basic knowledge about non-standard collembolan species, and their ecology, performance under laboratory conditions, and sensitivity to contaminants and soil properties.

1.3.3 Enchytraeids

Enchytraeids are small oligochaete worms, generally considered to be saprovorous and microbivorous, stimulating microbiological activity in soil through grazing and dispersion of spores.[94]

Some species are easy to rear and are cultivated as food for fishes by some ornamental fish breeders. However, basic ecological studies with non-standard species of enchytraeids remain poorly understood, which is caused by a lack of taxonomists, unfamiliarity with collection methods or even ignorance about this group.[95]

ISO guidelines[47] bring a list of potential species to be used in enchytraeidae reproduction test: *Enchytraeus crypticus* Westheide & Graefe, 1992, *Enchytraeus buchholzi* Vejdovsky, 1879, *Enchytraeus luxuriosus* Schmelz & Collado, *Enchytraeus bulbosus* Nielsen & Christensen, 1963. However, the most important criteria for species selection, besides basic knowledge about

culture requirements, is the ecological relevance and sensitivity to contaminants in comparison to the standard species *Enchytraeus albidus*.

Besides the standardized reproduction test, avoidance tests were proposed for the standard species *E. albidus*;[96] however, because of the lower sensitivity and higher variability, the enchytraeid avoidance test was not recommended for risk assessment purposes.[97]

Among the non-standard species, *E. crypticus* has been successfully applied in ecotoxicity tests around the world and has the advantage of good performance and speed of reproduction.[38] This species is sensitive to a range of contaminants and it can be used in risk assessment of contaminated sites.[98–100] Alternative endpoints using embryotoxicity tests were proposed for this species.[101]

Other species are mentioned in the literature, especially in studies with metal contamination: *Fridericia bulbosa* in lethality tests, *Enchytraeus doerjes* and *Enchytraeus bigeminus* in reproduction tests.[37,39,102–105]

Other aspects, such as the influence of soil properties on performance of enchytraeids and their interaction with soil contaminants, should be better understood.[106]

1.3.4 Isopods

Terrestrial isopods are saprophagous soil organisms that have a key role in litter fragmentation. The availability of studies about soil isopods, their ecological relevance and the ease of manipulating them make this group one of the most promising to be included in standardized protocols.[15,34]

No standardized species or guidelines exist at this moment for isopod tests. Among the challenges for their standardization are the high variability among individuals, probably because of sexual reproduction and the long timescale for reproduction in comparison to other invertebrate species, low reproduction rates, different sensitivities between males and females, and lack of knowledge about the basic ecology and life cycles of the species. Information on the methods to maintain and rear isopods in the laboratory is available for some species.[107–109] The main factors influencing isopods in culture include humidity, temperature, pH, and food quality.[110–112]

Porcellio scaber is one of the most used isopod species in ecotoxicity tests. Other species, such as *Porcellionides pruinosus, Porcellio laevis, Porcellio dilatatus* (Figure 1.2C), *Armadillidium vulgare*, *Cubaris murina* and *Oniscus asellus*, are suitable for ecotoxicity tests for a range of contaminants. Avoidance behavior tests were proposed for isopod species[113] and have been found to represent a more sensitive endpoint than lethality or sublethal endpoints,[114] suitable for screening of contaminated sites.[115] However, their tendency to aggregate could be a challenge for avoidance tests, because this behaviour might lead them to choose sub-optimal conditions.[114]

Lethality is considered a low sensitive endpoint because isopods can decrease food consumption (= intake) and tolerate high concentrations of contaminants in the environment.[116,117] Biomass loss is one promising

endpoint for ecotoxicity tests because of its great sensitivity and ecological relevance,[118] since weight loss is related to lower reproduction and consequently lower success of populations in the environment.

Reproduction tests are proposed with the exposure of non-gravid females,[119] as well as with exposure of truly gravid females.[120] Other endpoints have been proposed, such as food consumption determined by faecal production rates,[121] bioaccumulation,[122] feeding behaviour.[123–125]

Enzymatic biomarkers have been evaluated in tests with isopods to help to understand chemical stress modes of action, but some authors have shown high levels of inter- and intra-specific variability.[126,127] Cell membrane damage by direct contact or by lipid peroxidation in *P. scaber* is caused by the ingestion of titanium dioxide nanoparticles.[128] Effects of endocrine-disrupting compounds on the molting regime, growth and protein expression in different organs of isopods have been studied.[129,130]

In general, a substantial body of experience should be obtained with biomarkers in isopods in order to facilitate their application.[131] Furthermore, improvements are necessary to optimize the already developed ecotoxicity tests, aiming at proposing a guideline with this important group for soil ecosystems.

1.3.5 Others

Studies with soil invertebrates besides those cited above are scarce. Among the groups of non-standardized species that have been used are mites and beetles.

Mites are abundant soil organisms involved in decomposition of organic matter, in nutrient cycling, trophic structure and dynamics in soil. Among mites, *Oppia nitens* is a good candidate species for a standardized test design, with adult survival easily assessed in a relatively simple design. A long-term reproduction test with *O. nitens* will require the use of a synchronized population and, on occasion, organic matter amendment when testing soils with low organic matter content.[132]

Beetles of the family Scarabaeidae are important organisms that promote the decomposition of the dung pat, destroying possible habitats for cattle parasites, allowing the release of nutrients into the soil and for plant growth, and acting as food sources for insectivorous birds and mammals.[133,134] That is why it is so important to study the effect of veterinary products on these species. A test with the temperate dung beetle *Aphodius constans* was developed[135] evaluating the survival of beetle larvae in 3 week duration tests with fresh dung as the substrate. The larvae were exposed to four veterinary parasitical pharmaceuticals (ivermectin, moxidectin, dicyclanil, and praziquantel) representing different treatment regimes, modes of action, and effect levels. This test was recommended for standardization in an international ring test for risk assessment of veterinary pharmaceuticals.

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CHAPTER 2

Higher-tier Multi-species Studies in Soil: Prospects and Applications for the Environmental Risk Assessment of Pesticides

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2.1 Introduction

The soil ecosystem hosts a tremendous diversity of organisms, from microorganisms to insects to mammals. While offering stable and constant conditions with slow turnover rates, it responds sensitively to disturbances, resulting in long recovery periods. Soils worldwide suffer from intense land use by agriculture and urbanisation.

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This chapter will emphasize the importance of soil organisms, providing ecosystem services of major importance in agricultural ecosystems (comprising in-crop and adjacent off-crop areas). Knowledge of the components of soil's biodiversity is essential in defining exact protection targets for Environmental Risk Assessments (ERA) for specific pollutants, such as biocides, pesticides and industrial chemical compounds. We define why the protection of populations and communities in soil could help to assure the long-term fertility of soils and to provide structured soils with natural water holding capacities (Section 2.2). In this context, we outline what the legislative demands from ERA of plant protection products (PPP) in the European Union (EU) are, aiming at an adequate description of all risks for the environment associated with long-term and intense use of various compounds in the environment, and how it is currently conducted. The described aim requires test systems delivering information on direct and indirect effects on diverse communities of soil organisms. In a tiered risk assessment approach, those should be complex multispecies test systems at higher-tiers. They give the opportunity to measure various endpoints still showing interactions between different levels of organisation. It is agreed that higher-tier multispecies studies like Terrestrial Model Ecosystems (TME) or full-scale field studies allow for the detection of effects on soil organisms (mainly belonging to the size class 'mesofauna', see Figure 2.1) with sufficient statistical power while being representative and protective for a large part of real field situations.

Two directions in ERA can be addressed by multispecies tests: Firstly, complex organismic endpoints can be considered, assuming that the ecology of species triggers the specific risks resulting from different exposure probabilities, inherent sensitivity and recovery potential. Secondly, it is shown that relocation processes of PPP can be manipulated and modelled in

Figure 2.1 Soil organisms can be classified by their size. Two representatives of the mesofauna, the collembolan *Isotoma anglicana* (Lubbock, 1862; right) and the oribatid mite *Acrotritia ardua* (C. L. Koch, 1841; left) measure between approx. 0.5 and 2.5 mm in length (photographs by A. Toschki).

experimental semi-field systems in high resolution, leading towards more realistic exposure scenarios.

The core question of a future ERA for soil organisms is how a tiered risk assessment approach could better accomplish the general goal of protection of biodiversity, even when extrapolating from lower tiers to the real world. Multi-species studies could be used as the surrogate reference tier as long as all tiers of the system have been adequately calibrated.

2.2 Ecological Relevance of Soil Organisms in Agro-ecosystems

The composition of inorganic and organic soil structures provides a medium for a variety of organisms, which use the soil as a habitat and as a source of energy. At the same time, soil organisms contribute to the formation of soil structures by influencing the soil's belowground and aboveground processes. Soil organisms suffer from agricultural practices to a great extent, and thus it is of particular importance to define overall concepts for agricultural landscapes that define site-specific soil organism communities.

2.2.1 Structure and Function of Soils and Soil Organism Communities

The structure and function of soil biocoenoses can be distinguished.[1–3] Structure refers to the composition of the soil biocoenosis (biodiversity). It can be described at the population or community level (*i.e.* as presence/absence, abundance, biomass, diversity and dominance), whereas functions refer to the interactions of different components of soils (*i.e.* nutrient cycling, community respiration, organic matter breakdown).[4]

The most dominant groups of soil organisms, in terms of numbers of individuals and biomass, are microorganisms, *i.e.*, bacteria and fungi. Besides, soil ecosystems contain a large variety of animals from various feeding guilds, like protozoa (bacterivores, omnivores, predators), nematodes (bacterivores, fungivores, omnivores, herbivores and predators), micro-arthropods, such as mites (bacterivores, fungivores and predators) and collembolans (fungivores and predators), enchytraeids and earthworms (both being mainly saprophagous). In addition, a high number of macrofauna species (mainly arthropods like beetles, spiders, diplopods and chilopods and insect larvae, as well as snails) live in the uppermost soil layers, the soil surface and the litter layer. All these organisms at different trophic levels and groups build the soil food web (Figure 2.2). Lots of information is available on estimating the abundance and biomass quantities of soil organisms in agricultural landscapes.[5]

Typically, soil organisms are distributed in a vertical gradient depending on soil litter input, as well as on moisture and temperature conditions. However, the top soil layer, often the uppermost centimetres, contains by far

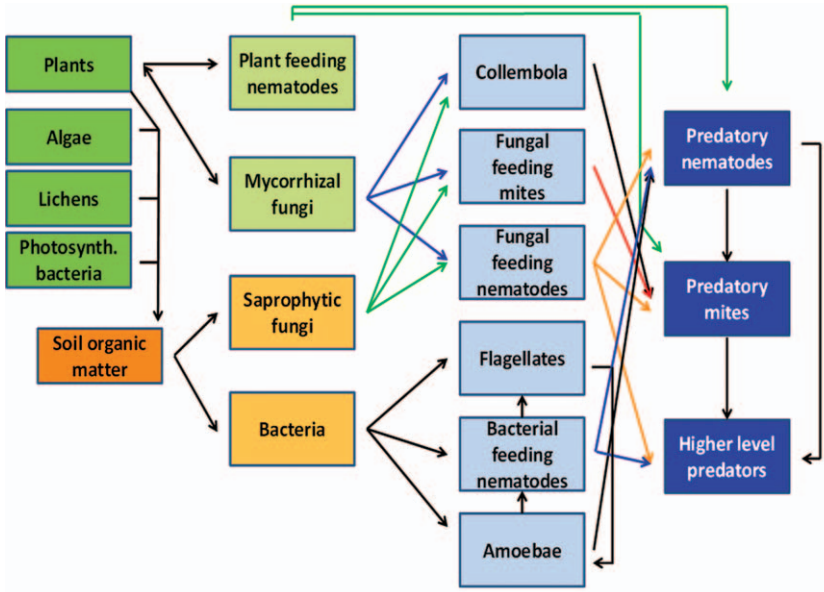

Figure 2.2 Partial structure of the soil food-web.

the most soil organism species and individuals. In grassland soils, it has been shown that more than 90% of the soil organisms—except for anecic earthworms—are present in the upper 5 cm layer.[6]

One of the most important soil functions is the decomposition of plant and animal debris and the formation of stabilized soil organic matter (humus). This process can occur over longer or shorter periods in soils with natural litter input but also under agricultural conditions with regular periodic soil cultivation activities and tillage.[7] This activity is fundamental to provide the fertility of soils over long periods and is essential for sustainable agriculture. Nitrogen fixation and organic matter breakdown are the backbone of nutrient and element cycling with a direct impact on the gas composition of the troposphere. Nitrification and denitrification processes as well as aerial and fertilizing loads of nitrogen will determine the pH of soils. In addition, anthropogenic chemicals, including pesticides but also pharmaceuticals, endocrine disruptors and other synthetic chemicals, can be degraded in soils preliminarily by bacteria and fungi, but they may have an impact on the degradation potential of soil itself. The physiology and behaviour of soil organisms contribute to the formation and stabilization of soil aggregates and structure. The pore system of soils, resulting largely from soil organism activities, is important for water, nutrient and gas exchange, crucial for all typical soil processes, and provides habitat niches for soil microorganisms. In addition, the soil food web provides biomass for the higher trophic levels in the aboveground ecosystem.

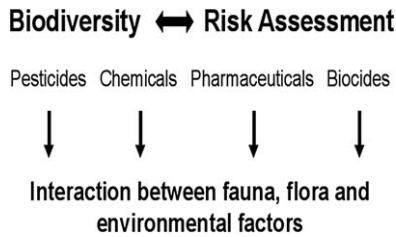

Figure 2.3 The relationship of environmental stressors and biodiversity must be accounted for in several domains of ERA.

2.2.2 Losses of Soil Biodiversity in Agricultural Landscapes

Biodiversity is the basic resource maintaining and supporting ecosystem services and functions. Biodiversity is realized at different levels of the complexity of a given landscape. It is widely accepted that species diversity and habitat quality dramatically decrease with increasing intensity of agricultural land use. In recent years, indeed, a constant decline of the biodiversity of agricultural landscapes has taken place, indicated by a severe decrease of, for example, numbers of bird and butterfly species and individual numbers.[8] Therefore, we lose a variety of benefits that are provided by the species. Gascon *et al.* conclude that the links between individual species and ecosystem services are direct, complex and often unexpected.[9] This clearly shows that current risk assessment was not protective enough over the last few decades to maintain the integrity of the biodiversity and the related functions. In particular, agricultural soils suffer from drastic changes compared with natural systems and soil communities do experience a range of different stressors.[7] Environmental stressors (like pesticides) act on the landscape level and related adverse effects are considered within national as well as EU legislation (Figure 2.3).

2.3 *Status Quo* and Developments of Risk Assessment for In-soil Organisms

2.3.1 *Status Quo*

The risk assessment of PPPs for in-soil organisms in the EU is a prospective ERA, which is currently undergoing a phase of transition. With the replacement of Council Directive 91/414/EEC[10] by Commission Regulation (EC) No 1107/2009[11] in 2011, a process of reviewing and updating data requirements and guidance documents started. It was not completed until the year 2016, and thus the 'Guidance Document on Terrestrial Ecotoxicology under Council Directive 91/414/EEC'',[12] which dates back to the year 2002 and refers to outdated regulation and data requirements, is still the leading harmonised document for the member states of the EU.

The basic principle of the risk assessment is a tiered approach, starting from hazard identification and characterization using standardized ecotoxicological laboratory tests with single species and environmental exposure predictions assuming worst-case conditions. The aim of these standardized laboratory tests is to describe the intrinsic toxicity of the test substance by either threshold concentrations (No Observed Effect Concentration (NOEC) approach) or dose–response relationships (Effect Concentration (EC_x)-approach). The exposure prediction is based on characteristic substance properties (*i.e.* persistence, $\log K_{OW}$) using methodologies developed by the FOCUS working group and on scenario-based application patterns.[13,14] Toxicity (T) and exposure (E) are subsequently set in relation to each other in order to assess whether this toxicity to exposure ratio (TER) is above or below the TER thresholds laid down in the uniform principles (Commission Regulation (EU) No. 546/2011[15]), indicating an acceptable or unacceptable risk, respectively. The tiered approach is principally open to refinements, *i.e.* if under the assumed worst-case conditions in tier 1 a risk is identified, higher-tier assessments are possible. This could comprise refining the side of the toxicity evaluation by using test systems set-up under more realistic conditions (*e.g.* using natural test soils), as well as the side of the exposure calculations using refined scenarios.[11,12] The ERA scheme in general relies on the representative species concept of testing earthworms, soil- macro-organisms and soil microorganisms to account for several trophic levels capturing structural and functional endpoints. The underlying logic of the tiered approach is that the simplified tier 1 should be so conservative that when no unacceptable effects occur in single species laboratory tests under assumed worst-case conditions, no effects on the population should occur. The relevance of those laboratory test results for the real world, however, has to be extrapolated with a high degree of uncertainty. This uncertainty is supposed to be accounted for by appropriate assessment factors, which are laid down as TER thresholds in the uniform principles.[15] It has to be noted, though, that within the current risk assessment scheme, it is not clear how the laboratory and the field test situation actually relate to each other, *i.e.* there is no calibration behind the current TER threshold.

2.3.2 Transition

The standard data requirements have already been modified during recent years. At the time when the current guidance (SANCO/10329/2002 rev 2 final[15]) was developed, only data on acute toxicity on earthworms (OECD 207[16]) and on nitrogen and carbon transformation (OECD 216, 217[17,18]) were mandatory. Data on effects on the reproduction of earthworms or other soil macro-organisms as well as the functional test on the degradation of organic matter in litterbags were only triggered under certain circumstances.[12] The acute test on earthworms turned out to not effectively identify problematic substances and has now been skipped with the most recent data requirements. The earthworm reproduction test (OECD 222[19]), and the collembolan and predatory mites reproduction tests (OECD 232[20] and OECD 226[21]) have

become mandatory instead, whereas the functional carbon transformation test is dismissed under Regulation (EU) Nos. 283/2013[22] and 284/2013,[23] putting a clear emphasis on species-level tests by doing so. For refined assessments, the guidance is limited.[12] Currently, the only higher-tier test system delivering structural endpoints and for which a harmonised test guideline exists is the earthworm field test.[24] The litterbag test is criticised as it is solely a functional test and, as such, it does not give any indication if and how the microbial community might have changed. Thus, it does not seem to be suitable as a refinement option anymore; in particular, in light of Commission Regulation (EC) No. 1107/2009, which requires it to be shown that a PPP has no unacceptable effects on non-target species, their behaviour, biodiversity and the ecosystem, which consequently comprise indirect effects and impacts on the food web.[25]

In the transition phase of regulations and guidance for many PPPs and active substances, data according to the old data requirements are still available and considered for the risk assessment. New substances and PPPs consisting of such have to be evaluated according to the new data requirements and all PPPs have to be evaluated according to Commission Regulation (EC) No 1107/2009. With the shift of regulations, an increasing number of PPPs do not pass tier 1 owing to the mandatory testing of collembolan and predatory mites. Authorities therefore faced a multitude of refinement approaches provided by applicants trying to prove that under conditions that are more realistic, the risk would be acceptable. These comprise, for example, multi-generation collembolan laboratory studies or collembolan population modelling. Thus, development of guidance that incorporates the overarching protection goals and state-of-the-art techniques into the scientific-based risk assessment is urgent to prevent non-consistent decision-making.

2.3.3 New Developments

Between the publication of the terrestrial guidance document (SANCO/10329/2002 rev 2 final[15]) and today, a series of activities has been undertaken identifying critical points of the existing risk assessment and targeting future development and harmonisation.

The starting point was the European Food Safety Authority (EFSA) public consultation on SANCO (10329/2002) that *inter alia* identified the lack of guidance on semi-field tests, and called for better guidance on how to interpret earthworm field data and functional tests, as well as for better linking of exposure to effects. It was also pointed out that the question of bioavailability in standard tests is not yet considered adequately.[26] Further authority activities comprise contributions for predicting environmental concentrations[14] and resetting data requirements for substance approval,[23,24] as well as on summarising the scientific expertise on drawbacks of the current approach regarding bioavailability of active substances, the appropriate level of protection for the environment, the lack of guidance on higher-tier options for species others than earthworms and on their interpretation.[27]

Concurrently to these activities, several workshops involving stakeholders from industry, academia and national authorities have been held, *e.g.* the PERAS workshop on semi-field test systems,[5,28] which identified terrestrial model ecosystems (TME) as suitable tools and gave recommendations on how to conduct TME studies. The German Federal Agency (UBA) organised a workshop on multi-species test systems,[29] where besides TMEs test designs for soil macro-organism field tests were also discussed. Yet even though experience with and publications on these test systems are increasing, up until now harmonised guidance on testing and test evaluation is only available for earthworms, comprising the commonly accepted recommendations on earthworm field testing[30] that amends the ISO guideline[24] and the guidance on summarising earthworm field studies developed in The Netherlands.[31] The progress made in translating available research results into operational guidance for in-soil organisms is still rather limited, especially when it comes to the calibration of the risk assessment scheme. That many questions remain open or are at least discussed controversially became obvious at the topical workshop of ECHA and EFSA in October 2015, which highlighted common problems in soil risk assessment under PPP, biocides and REACH regulations, and where hardly any new topics than the aforementioned were raised.[32]

2.3.4 Challenges

Developing a straightforward yet adequate risk assessment scheme for in-soil organisms is a challenging task for the authorities. While the impact on single species can be surveyed and assessed precisely in standardized laboratory systems, the impacts on populations and communities are more challenging to assess. Field tests are the test systems that are most close to the real field situation. Yet while the realism of complex field studies is comparably higher, the reproducibility of the results is lower owing to inherent variability in the field and it is necessary to adapt the methods to the higher variability to get precise results. As described above (Section 2.2) many different factors affect biodiversity in arable landscapes (*i.e.* land use, fertilisers, pesticide use, and crop type). In order to control for these influencing factors, co-stressors are typically minimized in semi-field and field tests, focussing on the effect of one individual PPP at a time, thus again ending in a less realistic situation than in the actual field situation. It is known, however, that changes in species communities are triggered by stressor-complexes.[33–36] Consequently, preserving the environmental quality up to a certain standard requires an integrative landscape approach.[33,37] In the context of a pesticides ERA, the different scales of ecotoxicological testing—landscape, semi-field and laboratory—as well as the methods used should be concerted (Figures 2.6 and 2.7).

Recent publications by EFSA suggest that future risk assessment for pesticides will move towards a more integrative consideration of the environmental conditions in the agricultural landscape.[25,38]

Several knowledge gaps are currently impeding an integrative assessment:

2.3.4.1 Exposure

- *Predicted Environmental Concentration (PEC) calculation*
  In laboratory studies with single species, typically nominal concentrations are given for homogenized soil. In the field, a pesticide is usually sprayed on the soil surface, resulting in a vertical concentration gradient. The real exposure of soil-living organisms varies additionally owing to the life-form type and behaviour of the organism.[39] The actual exposure depends on and varies gradually with the soil layer that is analysed; it also varies over time and is influenced by the bioavailability of the substance.
- *Mixture toxicity and application scenarios*
  In the laboratory, usually one pesticide is tested at a time. Under real conditions in the landscape, organisms are exposed to a mixture of several pesticides. Additionally, serial applications of pesticides and their mixtures must be assumed as normal.[40]

2.3.4.2 Effects

- *Species interactions and population dynamics*
  In the laboratory, natural species interactions (competition, feeding *etc*.) are not captured. In addition, the population dynamics are not considered in most of the cases. Indirect effects caused by the loss of competition or feeding resources within the community are crucial in population development.[41] When integrating modelling approaches into the ERA, these aspects have to be considered.
- *Species behaviour and spatial distribution*
  In the arable landscape, species are not distributed evenly in the soil, neither vertically nor horizontally. Consequently, the behaviour and distribution preferences of species are highly relevant to determine their exposure and to calculate and assess risks.

2.3.4.3 Linking Exposure and Effects

There is currently a lack of described interrelations between the lab toxicity testing and the corresponding effect patterns in the field,[6] hindering a proper calibration of the tiered approach.

2.3.5 Future Demands

Several regulatory domains, such as pesticides regulation, chemicals registration, soil conservation, pharmaceutical registration and nature conservation, account for ecological effects and have to be conducted in relation to the same overarching protection goal, whereas the effect has to be assessed

in each field monocausally in relation to the individual stressor (that is, the specific exposure). Since biological communities are responding to combinations of environmental stressors,[33,35,36] it is essential to regulate the environmental quality in agricultural landscapes based on a landscape-related, overarching integrative approach. Even if the intensity of pesticide use is reduced in the future, an automatic recovery of many populations on the landscape level cannot be expected. Every habitat type bears a typical adapted community of species. For example, in-field communities differ from off-field communities, and grassland communities from those of cultivated crops.[33,42] Whereas some species are interrelated, crossing different habitat types, and can be interchanged, some cannot and exchange is not possible. The dispersal potential is limited for most species.[25] Additionally, in arable landscapes the area of cultivated land is by far higher than that of off-crop habitats (approx. >80% *vs.* 10%).[33] The potential for external recovery is thus very limited and the potential for internal recovery has to be reflected against the backdrop of the presence of multiple stressors.

The ecological value of agricultural landscapes should be actively improved. To achieve this goal, overall concepts have to be defined to set the best case of agricultural practice in cultivated landscapes comprising information about the regional potential of biodiversity. Consequently, agricultural habitats in a poor ecological state (comparable to the Water Framework Directive or the concept of the Habitats Directive) should be reinforced to achieve a good ecological status as a kind of quality improvement. Development targets should be defined that deliver definitions of an acceptable range of typical communities for the agricultural landscape on a regional level.

Test systems should contain communities that are able to integrate indirect effects affecting the interaction between species.

2.4 Methodologies for Multispecies Tests in Soil

From the latest developments of in-soil risk assessment after the conversion of the original directive 91/414[10] into the new pesticide regulation 1107/2009/EC,[11] functional endpoints become less important in favour of structural endpoints. These are better suited to addressing the main issues concerning biodiversity parameters than the few available functional tests (microbial enzyme activities, litterbags, bait-lamina sticks).

2.4.1 Ontology and History of Test Systems

The first attempts to develop and standardize ecotoxicological test procedures were invented by the IOBC/WPRS ('International Organization for Biological and Integrated Control of Noxious Animals and Plants'—Working Group on Pesticides and Beneficial Organisms). The main aim of the group was to have methods at hand that allow for the description of undesired side-effects of pesticides on beneficial organisms. From this starting point,

experimental multispecies approaches in soil have a relatively short history back to the 1980s. Multispecies test systems were used both for ecological and ecotoxicological research questions. Those systems have at least one characteristic in common: they offer bounds towards surrounding biotic and abiotic influences.[43] Depending on the authors involved, semi-field systems were termed 'mesocosms',[44] 'semi-field enclosures',[45] 'soil microcosms',[46] 'terrestrial model ecosystems',[47] 'soil multi species—SMS' test,[48] 'MS3' test[49] or 'small-scale terrestrial ecosystem—STEM'.[50]

For classification of semi-field systems, the proposed nomenclature of Morgan and Knacker has proved useful.[51] They introduced four classes according to two main classifiers: Firstly, a test system may be open, allowing for atmospheric gas exchange, or closed. Closed homogenous systems do not allow atmospheric gas exchange and are mainly designed to follow the fate and behaviour of chemicals. Secondly, a system could be intact, containing undisturbed soil, or homogenous, containing sieved soil. Sieved soil was often defaunated prior to the experiments, followed by addition of a few species (category A). Closed intact systems do not allow for gas exchange as well. However, they were mainly designed to demonstrate the effect of or on natural soil organism communities (category B). Open homogenous systems allow for atmospheric exchange, a feature that makes them unsuitable for fate studies. The sieved soil was often defaunated (*e.g.* by freezing the soil), followed by addition of a few species (category C). The last category (D) comprises open intact systems (atmospheric exchange possible) containing undisturbed soil cores and thus allows for the description of effects on (initially) natural soil organism communities. A mixed category remains for publications that combined various methods, such as combinations of one of the types A–D with each other or with field studies. This category also covers field enclosures, mainly mesocosms, as mentioned above, a prominent system during the 1990s.

Tracking the number of referred categories over the period of three past decades, the trend points towards more intense use of open systems, which can contain sieved or intact soil in equal measure (Figure 2.4). Nowadays, depending on the research questions and the intended level of complexity, open systems with various conceptual approaches are in use. On the whole, they could be divided into (re-) assembly and perturbation experiments, bearing in mind how (more or less recently) established mechanisms of interactions have to be interpreted and at which level of confidence to detect causal relationship on the one hand, and to be relevant for the field situation on the other hand.[52]

Most recent test systems use open semi-field installations; the approaches can be generally distinguished as intact, perturbed systems or sieved, assembled systems. The soil substrate of category C systems often consists of natural, standard soil (*e.g.* LUFA soil) that was often sieved to homogenous particle size, or of standardized, artificial substrates (*e.g.* following OECD guideline 207[16] for the acute testing of earthworms). The sizes of containers can be relatively small (15–20 cm in diameter, 30–40 cm in height), and the

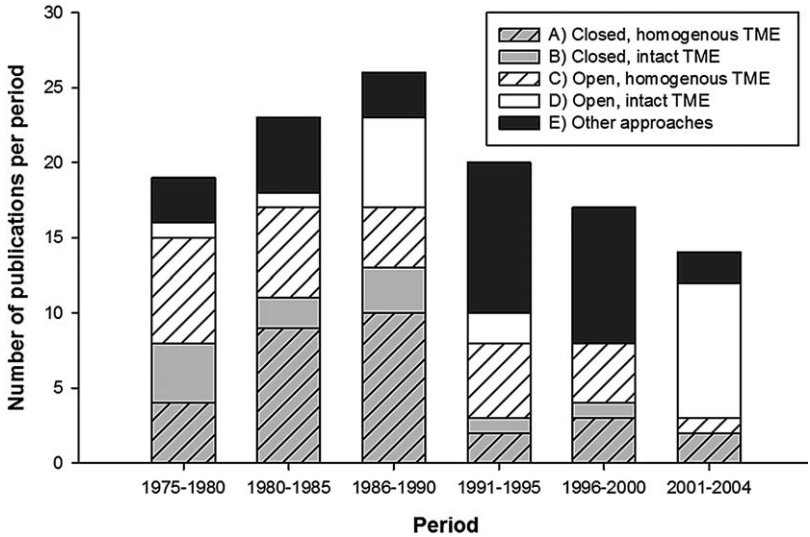

Figure 2.4 Semi-field study approaches. Bars sum up to 119 publications published in the last 30 years.

test organisms (plants, micro-arthropods, earthworms, potworms) often came from laboratory rearing, or were extracted alive from natural soils. The small size allow for high numbers of replicates and treatment levels; up to 100 systems have been used for a single study. The systems used by Boleas *et al.*[49] and Fernandez *et al.*[53] ran for 21 days and were equipped with natural communities of soil organisms and artificially added individual plants and earthworms.

Open, intact systems of category D, often referred to as Terrestrial Model Ecosystems, have been developed since the early 1990s and it was attempted to propose guidelines for the standardization of this kind of semi-field tests.[47,54,55] Those systems were usually larger than the sieved variants (diameter up to 45 cm, height up to 60 cm) and contained natural soil communities after being cored from agricultural or grassland soils.

Both approaches measure a wide variety of endpoints, from microbial activities,[56] over functional endpoints (feeding activity by bait-lamina sticks) to genetic (gene expression patterns by denaturing gradient gel electrophoresis[57]) and taxonomic community endpoints (*e.g.* principal responses of enchytraeid communities.[58]

The fate and behaviour of chemicals have to date mainly been investigated in closed systems. Thus, a complete balance of the degradation of a substance could be established. Very recently, the vertical relocation to lower soil layers of pesticidal chemicals was followed to determine the environmentally relevant concentration in soil.[6]

Figure 2.5 shows the development of the main research aims over the past decades. Most obviously, a shift from studies that focus on the fate and

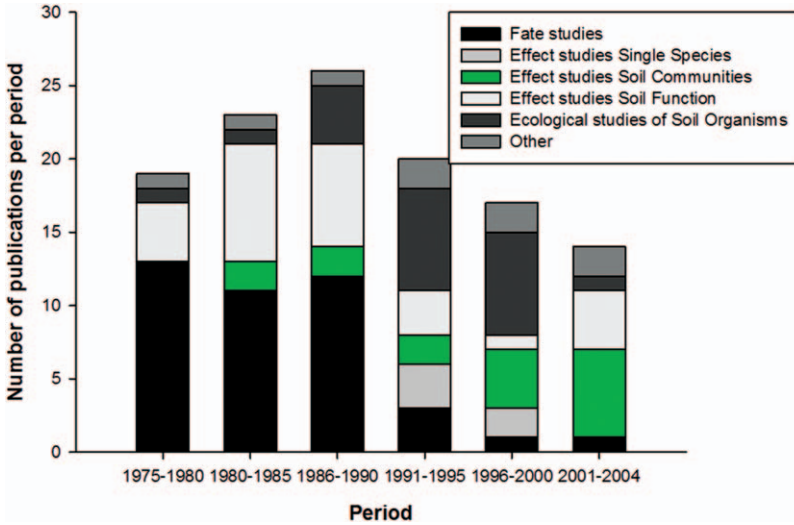

Figure 2.5 Research aims of semi-field study approaches. Bars sum up to 119 publications published in the last 30 years.

behaviour of environmental contaminants towards more effect-oriented studies, *i.e.* community studies took place.

After the discussions during recent workshops initiated by regulatory and academic bodies,[29,32] it has been widely agreed that intact, open TME containing complex communities better address biodiversity issues than assembled, homogenous systems.

2.4.2 Methodological Challenges of Multispecies Tests

TME proved to be sensitive and reliable ecotoxicological test systems[5,57,59] and suitable to fill the gap between laboratory and landscape level (Figures 2.6 and 2.7).

The method of TME allows the testing of intact natural soil communities and captures intra- and inter-specific competition as well as predation. Therewith, the detection of indirect effects and recovery is possible and a direct link to realistic field conditions is given. The natural community of different soil organisms (collembolans, mites, nematodes, lumbricids, and enchytraeids) can be assessed on a community level as well as on a population level over a period of time (Figure 2.8).

For both cases, the MDD (Minimum Detectable Difference) can be calculated as a measure of precision and used to check the relevance of 'no-effect' findings. The methodological criteria given by Brock *et al.*[60] proved to be valid for different organism groups in different TME studies (Figure 2.9). With a reasonable test design, the derivation of ecotoxicological values, *e.g.* NOEC or EC_x, is possible.

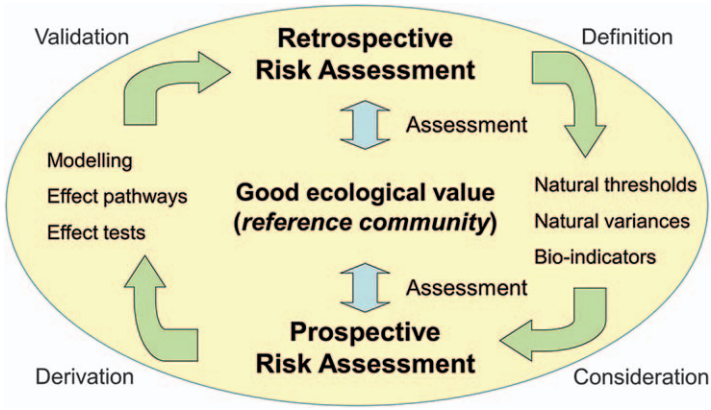

Figure 2.6 Interrelation between the different scales in tiered ERA.[37]

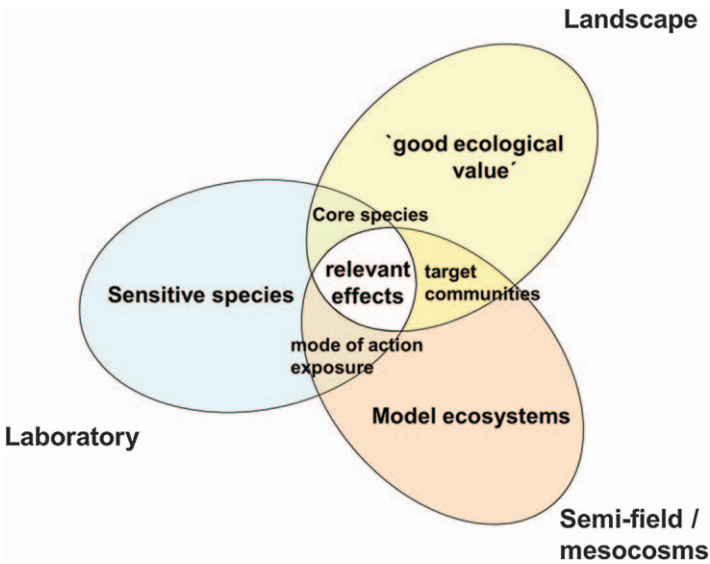

Figure 2.7 Integrative and iterative approach to interrelate the different approaches used in tiered ERA.[37]

On a European level, TME have been recently discussed to be a reference tier to calibrate risk assessment[27,32] (discussed in Section 2.6). With TME, it is possible to survey exposure and effects of pesticides on different species groups at the same time.[39] Basic data can be recorded from natural communities, which are crucial to model population dynamics in the field. The beneficial characteristics for ERA of TME are listed as follows:

– Natural undisturbed community of soil organisms (collembolans, oribatid mites, enchytraeids, nematodes, lumbricids) can be surveyed under field conditions.

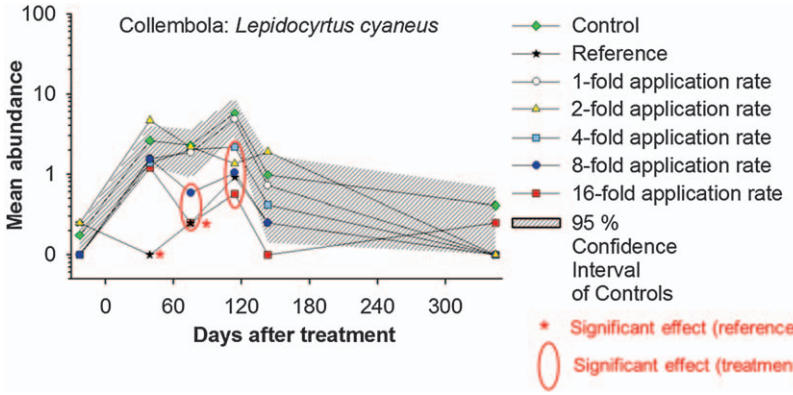

Figure 2.8 Dose-related effects of a pesticide on the collembolan species *Lepidocyrtus cyaneus* in comparison to a toxic reference substance and a control.[91] ©2009 Society of Environmental Toxicology and Chemistry (SETAC). Reproduced with permission.

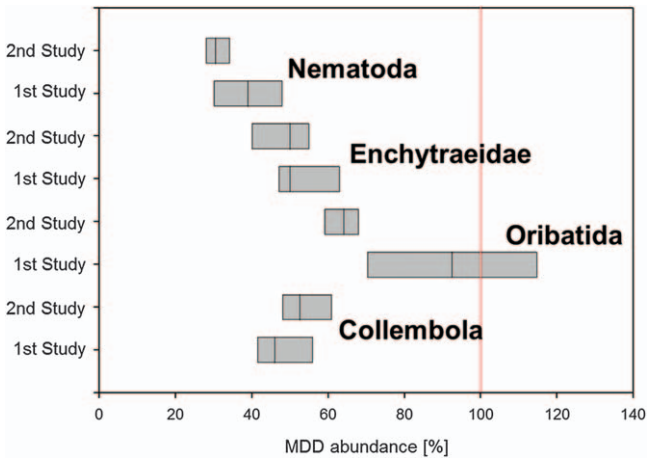

Figure 2.9 Calculated Minimum Detectable Difference (MDD) of abundance for two different TME studies and four different soil organism groups.[92] ©2014 Research Institute for Ecosystem Analysis and Assessment—gaiac. Reproduced with permission.

- Lower variances of abundances owing to sampling and small-scale coring of the TME.
- Sequential sampling up to 1 year after coring. The sequential sampling procedure led to a maximum lost surface area of 20% of a TME.
- Grassland TME can be used as a worst-case scenario for the in-field situation.
- The detectability of effects—expressed as MDD—on group abundances and for the least variable populations was smaller than 60%, effect detection classification (<50%).
- Exposure of pesticides and effects can be measured at the same time.

- Reduced variability of environmental conditions, standardised values can be set, *e.g.* irrigation, coverage.
- Possible measurement of percolation.
- Reduced land consumption.

2.5 Exposure of Soil Organisms Resulting From the Fate of Pesticides

After application of pesticides, certain amounts of the products will reach the soil depending on the application rate, the method of application (solid or liquid products, seed dressing), the vegetation status (soil covered *via* interception), and the weather conditions, especially wind (influencing spray drift) and rain (determining lateral and vertical washing-off and leaching, respectively).

The fate of the pesticide on or in the soil generally comprises three different processes: (1) transport, *i.e.*, leaching, run-off, and volatilization; (2) partitioning, adsorption and binding in the soil environment; (3) transformation, both by biotic actors, *i.e.* microorganisms and extracellular enzymes, and by abiotic routes, *i.e.* hydrolysis, oxidation–reduction, photolysis, catalysis on inorganic surfaces, such as clay and metal oxides, and the presence of reactive oxygen species, like hydroxyl radicals.

Solid adsorbents for the pesticides are the different soil constituents that may exert several adsorption mechanisms: hydrogen bonding, ion exchange, and complexation with metallic cations, polar and hydrophobic interactions, charge transfer, and Van der Waals dispersion forces.

The rates of transformation and degradation depend both on the properties of the pesticide molecule and of the soil. With regards to the chemical properties, most important are the water solubility and lipophilicity, often estimated by the so-called octanol–water partition coefficient, structural characteristics, such as the readily cleavable functional groups (*e.g.* esters) and the degree of branching determining the adsorption and the inherent (bio)degradability, the Henry coefficient, which describes the distribution into the atmosphere, and acid–base constants of ionisable groups. The half-life of degradation, $DegT_{50}$, has to be distinguished from the dissipation half-life, $DisT_{50}$, the latter simply stating that after a certain time only half of the molecule can be analysed after extraction of the soil. This is not equivalent to degradation *per se* because often major parts of the chemicals form non-extractable residues (NER), which in most cases remain a black box because the identity of the residues and the binding mode are unknown (see below). Most often, first order kinetics, sometimes with multi-compartment adjustment, are assumed to describe the disappearance of a pesticide in soil.

Important soil characteristics that determine the fate of a pesticide are the organic matter content, the texture (especially the clay content), cation exchange properties and the soil moisture, which triggers the microbial

activity of the soil. Soil moisture affects the diffusion of the pesticide molecules and the mobility of microorganisms at lower soil water contents, while at high moisture levels, processes may be limited by oxygen availability: both will affect the activity of soil microbial communities. To some extent, the soil pH is also important if the pesticide is hydrolytically labile or because specific soil enzymes are pH-dependent. A wealth of literature is available describing the interdependent and multi-scale factors that determine the fate of chemicals in soil.[61,62]

The degradation of chemicals in soil leads to the formation of extractable, volatile, and non-extractable metabolites. The amounts of extractable and volatile metabolites can be structurally elucidated by spectroscopic and spectrometric techniques depending on the quantity available for analysis. NER analyses, however, depend on (stable or radioactive) isotope labelling. Chemical derivatization techniques allow a further distinction of NER types comprising xenobiotic residues entrapped in the voids of the inorganic and organic soil matter (type I), those covalently bound to humic material (type II), and biogenic residues (type III) such as amino acids and fatty acids formed by microorganisms that can use carbon or nitrogen from certain pesticides for synthesis of cell constituents. After death and cell lysis, such compounds incorporate into soil humic matter, ultimately forming biogenic residues. For the ERA of NER, the potential subsequent release of non-extractable parent substances and xenobiotic metabolites, especially from type I NER, should be taken into account, as recently discussed.[63] On the other hand, biogenic (type III) NER, formed by readily biodegradable chemicals are of no environmental concern.[64–67] Thus, despite the low amount of humic matter in soil, it is of great importance for the fate of pesticides.

It has to be taken into account that climatic conditions may have a strong influence on the fate of pesticides in soil, *i.e.*, temperature and precipitation, and thus on the exposure and sensitivity of soil organisms.[68,69] In addition, further soil amendments like sewage sludge application for fertilization may influence the fate of pesticides.[70] What is not considered at all in determining the fate of pesticides, so far, is the presence of several active ingredients owing to serial applications or to the use of multicomponent products. In most agricultural crop protection scenarios, several active ingredients are involved. One may argue that a certain fungicide may exert toxicity on soil fungi and correspondingly may decelerate the degradation of another pesticide, as observed by Swarcewicz and Gregorczyk.[71] On the other hand, adaptation of the soil microflora may enhance the degradation of pesticides if other, similar, active ingredients have been applied before.[72,73]

Many of the parameters discussed so far can be combined to model predicted environmental concentrations (PEC^{14}) in soil, assuming homogeneous distribution in the top soil layer, 1 or 2.5 cm for lipophilic compounds with a high tendency to sorb to the soil matrix, or lower depths, *e.g.* 5 cm, for less lipophilic substances. Soil organisms living preferentially in the top soil layer are usually exposed to rather high concentrations of lipophilic substances, which sorb readily to soil organic matter and do not

move to deeper soil layers, except that leaching is promoted by binding to dissolved organic matter[74] or particulate matter[75] or preferential flow paths.[76,77] However, so far the movement of soil organisms in the soil profile has not been considered when assessing their exposure. Soil organisms like endogeic earthworms that prefer deeper soil layers will still temporarily be exposed in the upper layers with high pesticide concentrations.

Methods to assess the bioavailability of pesticides in soil include extraction with aqueous solvents[78,79] assuming that the residues extracted under such conditions could be taken up by organisms from the soil pore water. Alternatively, solid-phase micro-extraction has been shown to reflect the bioavailability of pesticides by comparing the amount taken up from soil pore water in specially coated microfibers and that resorbed in soil organisms.[80–82]

2.6 Calibration of Soil Risk Assessment Using Semi-field Studies as Surrogate Reference Tiers

The ERA for chemicals of various purposes (industrial chemicals, PPP, biocides, veterinary and human pharmaceuticals) consistently depend on precisely defined protection goals for each subject area of protection. In agriculturally used soils, the general protection goal 'the biodiversity of soil organisms has to be maintained effectively over long periods' should be applied according to applicable law.[11] This overarching goal has to be refined and concretized in order to reliably predict the potential effects of accidental release or purposeful application of pesticides and to make the ERA procedural. In a tiered approach, all levels should address the same specific protection goal, reflected by TER thresholds being higher for lower tiers (assumed to be more conservative) and lower for higher tiers (assumed to be more realistic compared to the field situation). Calibration of the tiers means that if no or imperfect data for one of the tiers are available (*e.g.* the surrogate reference tier) its outcome can be interpreted in terms of its uncertainty, and predicted by using data from other tiers.[27]

2.6.1 Specific Protection Goals

In regulatory practice, concrete 'specific protection goals' must be defined. For a general goal of protection of 'biodiversity', the target area (either in- or off-crop), the acceptable deviation from the normal operating range or control level (*e.g.* as magnitude of effects between less than 50% of control level) and the time to full (internal or external) recovery has to be fixed. In terms of appropriate test systems, it must be possible to address the effect size by experimental and by modelling approaches to interpolate between all levels of biological organization and finally extrapolate to the real world. A testable, specific protection goal could be, for example, '*only negligible effects on the community structure of populations of soil organisms of more than one trophic level at field scale for an interval much shorter than the interval between two applications of a PPP should be expectable with high confidence in the result*'.

2.6.2 Derivation of Assessment Factors

In a risk assessment framework, it is not possible to test the effects of an application of a PPP directly in the real world with sufficient representativeness and accuracy. For the in-soil assessment, EFSA proposes the use of a surrogate reference tier that consists of experimental studies addressing indirect effects, population and community endpoints in connection with population models that address long-term recovery processes within the populations.[25]

For the calibration, *i.e.* the spreading between TER thresholds that indicate acceptable risks (or in other words the assessment factors), information is needed that could be used for statistical modelling of the variability in experimental data. The variability of measurements, the resulting uncertainty of the predictions for each tier and the representativeness of a particular test system compared to the 'real' world in a reference state have to be taken into account. In the EU, it has been proposed that the 'real world' could be divided into soil ecoregions that are considered relevant for the derivation of ecologically relevant concentrations (ERC) in soil.[83]

EFSA stipulates to list and estimate all imaginable and quantifiable sources of uncertainty and variability in a risk assessment procedure.[84] The overall uncertainty of the regarded tier can be derived by multiplying or summating the individual uncertainties from statistical modelling.[85] It has to be kept in mind that most assessment factors used today in different regulatory contexts are derived as rules of thumb, aimed at providing sufficient levels of conservancy in data-poor situations, and should be reviewed if new methods are implemented in the risk assessment.[86]

From a statistical point of view, the variability of measurement endpoints does not cause uncertainty. As long as the type of distribution and the characteristic properties of it are known (*e.g.* mean, standard deviation), probabilities for exactly defined confidence intervals could be given. Uncertainties come from lacking or incomplete information or from incorrectly asked questions that are not adapted to the target of protection. An assessment factor should help to deal with the problem that insufficient data has to be used for the extrapolation to a non-tested situation. The sources of uncertainty vary between the different levels of a tiered approach. Thus, strictly speaking they have to be assessed separately at each tier. The calibration of appropriate assessment factors has to be done for each extrapolation and multiplied (under the assumption that each of the uncertainties act simultaneously and independently on the outcome of the extrapolated protection level) if it is aimed at skipping one or more tiers (*e.g.* direct extrapolation from lab to real-world effects).

The *sources of uncertainty* for effect data come from the need for extrapolation, from lower to higher-tiers, from short- to long-term effects and from mono-species data to complex communities. They are difficult to address with exact numbers and can be only reduced by generating more appropriate data adapted to the core task of a risk assessment (*e.g.* protection of biodiversity).

The *sources of variability* for the toxicity data come mainly from variation between experimenters and taxonomists within and between laboratories and from variation between and within species. This variation is amenable to statistical evaluation. The number of sources of variability and the relative amount of variation in toxicity data usually increases from lower to higher tiers. In semi-field studies variation from climatic conditions or uneven distribution of the organisms in soil add up, whereas they are constant in laboratory experiments.

An analysis of the extrapolation of laboratory studies with few or even only one standard species to untested species of the same group of organisms or all soil organisms by a species sensitivity approach has shown for earthworms that the actual uncertainty factor of 10 was far from sufficient to cover the differences in sensitivity between species.[87]

2.6.3 TME as Surrogate Reference Tier

TME containing grassland soil communities reflect a diverse, undisturbed situation that could serve as a reference tier in an ERA, covering most effects on biodiversity expected in the field.[57,59,88] The protection level of lower tiers then has to be validated against the reference tier. Up to now, there are no systematic approaches that compare the protection levels reached by tier 1 or intermediate tiers with the reference level (from semi-field or field studies) for the soil compartment, as published for aquatic effect data.[89] It would be necessary to compile data on different classes of chemicals (*e.g.* PPP) for acute, chronic and (semi-)field tests. For the time being, first attempts with single substances have already been proposed.[90]

For cases where the lower tiers do not provide a sufficient level of protection concerning the actual specific protection goal, the assessment factors have to be adapted to a sufficient level (after calibration, Figure 2.10),

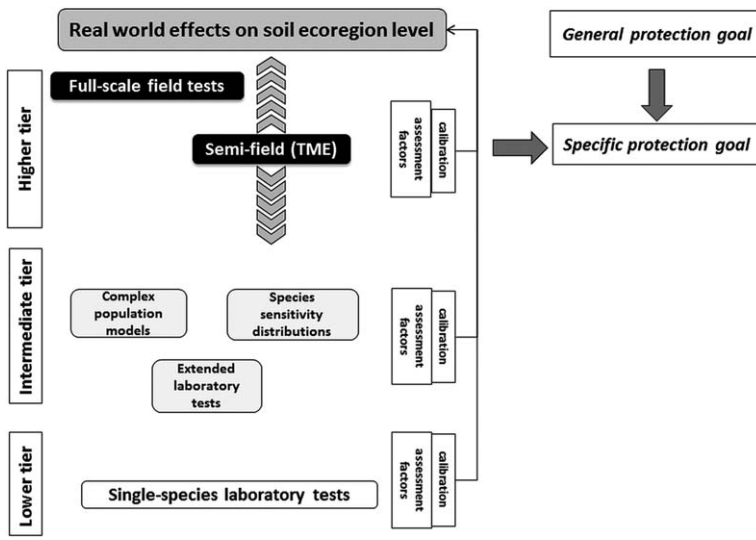

Figure 2.10 Calibration of the tiered approach for the in-soil assessment. Modified according to EFSA (2010).[27]

considering all factors affecting the remaining uncertainty of the risk assessment.

2.7 Conclusions

The ERA for pesticides in soil is in transition. The legal and regulatory frameworks are defining general and specific protection goals, being obviously aware that the ecosystem services of soils strongly depend on intact and diverse communities of soil organisms. Under this new perspective, methodologies are needed that robustly detect direct and indirect, acute and chronic effects on populations and communities. Overall concepts should be defined on regional scales, for the composition of soil communities in a 'good ecological status' as well as for the exposure assessment that largely depend on variations in intrinsic substance properties, soil characteristics and climatic conditions. Actual and future regulatory requirements and the long history of multi-species test systems in soil clearly show the potential of playing a key role in the prospective ERA of PPP. In addition, it gives a glimpse of the opportunities for multi-species test systems to add valuable information to regulatory areas (biocides, veterinary and human medicines, REACH chemicals).

One main attribute of all transitional processes that apply here is that many questions raised are not sufficiently answered so far. However, to meet the requirements of sustainable land use and the use of pesticides it is mandatory to recreate the recent risk assessment in a way that impacts on soil biodiversity can be measured in the field and the assessment must be adapted to the impacts and not to artificial beliefs.

The quantification of uncertainties and the deduction of appropriate assessment factors at each stage of a tiered approach has to be pushed by systematically analysing data that are available and by identifying crucial knowledge gaps. The definition of reference states of soil communities (the 'normal operating range') has to be conceptually formulated, defined by in-depth data analysis and then put into a broad scientific discussion. Basic questions that are still subjected to discussions touch the concepts of how to consider internal or external recovery. Completely open is the consideration of cumulative effects in ERA.

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CHAPTER 3

Aporrectodea longa *(Annelida, Lumbricidae): A Suitable Earthworm Model for Genotoxicity Evaluation in the Environment*

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3.1 Introduction

Aporrectodea longa is a relatively large species of earthworm (Figure 3.1), which makes it amenable to easy handling. It is typically found in open alkaline soils and is prevalent throughout regions such as England and western Germany. It is an anecic earthworm in that it has the ability to incorporate carbon from the surface to depth in soil.[1] In an ecological context and as saprophytes, they drive decomposition of organic material (*e.g.*, plant litter). However, this also brings them into contact with the vast range of environmental contaminants that move into the soil, so potentially they can occupy a role as a sentinel organism. *A. longa* is a mobile species and can

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Figure 3.1 Example of *Aporrectodea longa* (Annelida, Lumbricidae) found in typical soil sample in North West England.

disperse quite readily over large regions, although environmental influences such as climate change or contamination might influence this.[2]

Assessing contaminated land requires approaches to bio-monitor and characterise risk.[3] Earthworms facilitate pivotal interactions within ecosystems through the mixing and translocation of soil constituents, or by serving as a conduit for contaminants to predators at higher trophic levels.[4] Sentinel species are required to be ubiquitous, sedentary, abundant and sufficiently long-lived with the capacity to be reasonably tolerant to toxicants that bioaccumulate. *A. longa* (Ude) is abundant and widely distributed in fertile soils, including soils of restoration sites.[5,6] It is a comparatively large, slow-growing species that consumes large amounts of soil, much of which is deposited as surface casts, in creating an extensive burrow network. It also consumes dead organic matter taken from the soil surface. As a deep-burrowing surface feeder it is classified as an anecic species.[7] Thus earthworms may represent a potential biomarker of fluctuations in sub-lethal levels of environmental contaminants.[8,9]

Earthworms are the most frequently studied invertebrates with regard to the uptake and effects of organic pollutants, and it has been demonstrated that they are able to absorb organic contaminants.[10] They play an important role in the soil ecosystem, particularly through the action of mixing soil, and have been identified as important carriers of contaminants, which may bioaccumulate in higher predators.[11] These organisms have been employed previously to investigate pesticide toxicity to non-target biota, and there is some recent work that has been dedicated to the investigation of the lethal and sublethal effects of pesticide residues in soils.[10,12]

The principle concerns relating to the application of pesticides are that these potentially harmful chemicals are repeatedly and knowingly applied to soils.[10] Thus it is of great importance that, following their application, the fate and behaviour of these compounds, either singly or in mixtures, can be predicted.[13,14] The ultimate fate of these compounds is dependent upon the complex interaction of several biological, chemical and physical factors, which pertain to both the soil type and properties of the contaminant itself.[15] The understanding of such effects is fundamental, as these factors mediate the loss of a compound from the soil system. In addition to the loss

or removal of a compound from the soil system, various physical and chemical factors also affect the accumulation of a contaminant within soil biota, or its retention in the soil matrix as a result of sorption.[16]

One approach that has been employed to monitor the sublethal effects of pesticides on earthworms, with reference to genotoxicity, is the alkaline single cell-gel electrophoresis ('comet') assay.[17] The alkaline comet assay is a microelectrophoretic technique that allows the extent of DNA damage in the form of single-strand breaks (SSBs) to be visualised and assessed in individual cell genomes.[18] Sensitive to a broad spectrum of differing genotoxicants,[19] this technique has been used previously as a biomarker of sublethal genotoxicity in earthworms exposed to soil amended with a known genotoxin benozo[*a*]pyrene (B[*a*]P).[17] The technique can be applied to monitor DNA damage to any eukaryotic cell type, and previous studies have employed cells isolated by extracting earthworm coelomic fluid.[20–22] Indeed, this approach has been successfully applied previously to assess the comet formation in earthworms exposed to two pesticides.[23] Coelomic leucocytes (coelomocytes) isolated from earthworms maintained in contaminated soils exhibited significantly elevated levels of DNA SSBs as measured by the comet assay.[20,21]

As a model system, *A. longa* is potentially an ideal system to monitor contaminants in soil wherein it can either ingest or encounter *via* dermal absorption a vast range of environmental contaminants (Figure 3.2). Whilst there have been a number of studies that have looked at the sublethal effects of pesticides on the earthworm, little work has been performed to investigate the toxicity and genotoxicity of aged pesticide residues to earthworms. The key aims of this study were therefore to investigate the extent to which pesticide residues are genotoxic to non-target biota in the soil system, and then link this to pesticide residue extractability. In order to achieve this, the comet formation potential of a known genotoxin, benzo[*a*]pyrene (B[*a*]P), was compared to that of three commonly used pesticides with differing properties. In addition, the extent to which the extractability of one

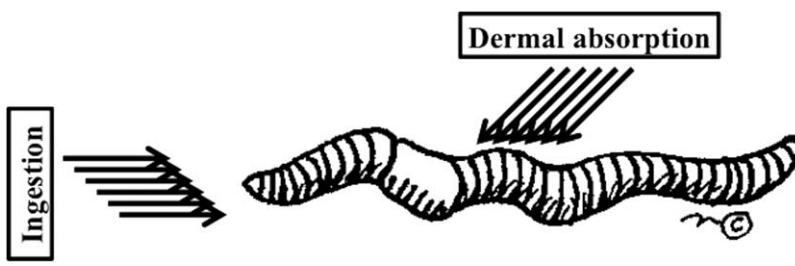


Figure 3.2 Simple schematic exhibiting the usefulness of the earthworm, including *Aporrectodea longa*, as a sentinel for environmental biomonitoring. Exposure may occur either *via* ingestion or trans-dermal absorption routes. Cells such as coelomocytes may then be harvested for incorporation into genotoxicity assays, such as the alkaline single cell-gel electrophoresis (comet) assay.

pesticide, cypermethrin, changed over time and how this might influence the formation of comets in earthworms was also investigated.

3.2 Experimental

3.2.1 Earthworm Collection and Storage

Specimens of *A. longa* were obtained commercially (Ecology Earthworms, Ipswich) or collected by excavation from neutral grassland on the Lancaster University campus. They were stored at 8 °C before use.

3.2.2 Soil Collection and Amendment

The soil used in this study was a loam to clay loam, with the soil samples having been collected from the top 5–20 cm of an organic pasture field, which had not received any pesticide or nutrient treatment for >20 years. The soil had an organic matter content of $2.7 \pm 0.04\%$ and a pH of 6.5 ± 0.08. Prior to spiking, the soil was partially air-dried, passed through a 2 mm sieve and rehydrated to 35% with deionised water (w/w).

Test agents (Sigma-Aldrich Co., UK) were dissolved in acetone and mixed into soil using the concentrated bolus technique.[24] Two sets of soils for all treatments were prepared Soils were amended with ^{12}C-cypermethrin only, to a final concentration of 0, 0.8, 8.0 or 80.0 mg kg^{-1}, for samples used for comet assay analysis. In addition, a duplicate set of soils was amended with ^{12}C- and ^{14}C-cypermethrin analogues for use in the extraction assays (see below). An initial 100 g of soil was placed in a glass mixing bowl, and the acetone containing each compound added and mixed thoroughly with a metal spoon for 5 min. The soil was then allowed to vent for 1 h in order to ensure the volatilisation of any residual acetone. Fresh soil was then added in two to three portions and mixed thoroughly to achieve a final concentration (mg kg^{-1} dry weight soil). Control samples were amended with acetone alone using the above procedure. All soils were then placed in sealed glass jars and stored at 18 °C for 24 h.

3.2.3 Exposure Following Amendment with Differing Pesticides or B[*a*]P

Duplicate samples (50 g) of soils that had been amended with ^{12}C compounds (B[*a*]P, cypermethrin, diazinon or isoproturon) only were placed in glass jars and adjusted to 35% gravimetric water content with deionised water. One earthworm (*A. longa*), which had been depurated for 24 h, was then added to each jar. After 24 h exposure it was removed from the soil and coeleomic fluid was collected prior to incorporation into the alkaline comet assay. Triplicate samples (50 g) of soils that had been amended with ^{14}C- or ^{12}C-cypermethrin analogues to a final activity of around 5000 dpm g^{-1} were treated identically with one earthworm added to each jar. Subsamples (1 g)

were removed before and after the addition of one earthworm to each jar (24 h) and used to assess extractability and for sample oxidation.

3.2.4 Coelomic Fluid Collection

Earthworm coelomic fluid was collected using a sonication method.[25] Earthworms were removed from soil treatments and rinsed with cold freshly prepared calcium-free *Lumbricus* balanced salt solution (LBSS) medium (1.5 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM $Na_2PO_4 \cdot H_2O$ and 4.2 mM $NaHCO_3$, adjusted to pH 7.3 with 1 M NaOH) to remove excess soil, and then placed in centrifuge tubes containing 10 ml of LBSS medium. The tubes were then placed in an ultrasonic bath for 3 s, around 10–15 times, until the solution became turbid.

Coelomic fluids were then centrifuged at 500 rpm for 5 min using a centrifuge (Beckman Centaur 2). The supernatant was discarded, and the pelleted cells were re-suspended in 1 ml of fresh cold LBSS medium.

3.2.5 The Alkaline Single Cell-gel Electrophoresis ('comet') Assay

The detection of DNA SSBs was achieved by alkaline lysis followed by alkaline gel electrophoresis.[17,19,26] Following extraction, each coelomic fluid suspension was mixed with 1 ml of 1% low melting point agarose (Life Technologies, UK), and 150 μl was applied to a microscope slide (Fisher), which had been pre-coated with 1.5 ml of 1% normal melting point agarose (Life Technologies, UK). Coverslips were immediately applied and the slides were then placed on a cold surface in complete darkness for 5 min. Coverslips were then carefully removed and the slides submerged in cold lysis solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris, 1% Triton X-100 and 10% DMSO, all supplied by Sigma-Aldrich Co., UK) and stored at 4 °C in complete darkness for at least 12 h. Slides were then rinsed in deionised water, transferred to a horizontal electrophoresis tank and covered in freshly prepared electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13, all supplied by Sigma-Aldrich Co., UK). The tank was then incubated at 10 °C for 25 min to allow unwinding of the DNA before electrophoresis at 0.8 $V\,cm^{-1}$ and 300 mA for 36 min, then removed, rinsed in deionised water and allowed to dry. The slides were then stained with aqueous ethidium bromide (20 $ng\,ml^{-1}$) and visualised by epifluorescence using a Leitz Dialux 20 EB microscope, with comet tail lengths (CTL; μm) measured using Comet Assay VI software (Perceptive Instruments, UK). A total of 100 images per treatment were analysed, which consisted of 50 each from duplicate slides produced from two specimens. CTL measurement data obtained from cell populations exposed to the pesticides were compared to control slides using a Mann–Whitney test.

3.2.6 Exposure to Aged Cypermethrin Residues

3.2.6.1 Soil Amendment and Sterilisation

Soils were amended with ^{12}C-cypermethrin only to a final concentration of 0, 0.8, 8.0 or 80.0 mg kg^{-1} for samples used for comet assay analysis. In addition, a duplicate set of soils was amended with ^{12}C- and ^{14}C-cypermethrin analogues for use in the extraction assays. All soil amendment was performed as described above. Immediately after amendment, the soils were sterilised by the application of a dose of >25 kgy of ionising radiation (Isotron plc, Bradford, UK). The soils were stored in the dark at 20 °C, and sampled after 24 h, 7 d, 30 d and 100 d. At each sampling timepoint, the soils were checked for sterility by plating out soil water extractions at 10^{-2} dilution onto sterile plate count agar plates incubated for 2 d at 25 °C ($n = 5$).

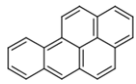
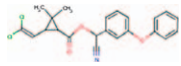
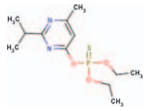
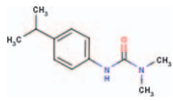
3.2.6.2 Extraction of ^{14}C-Associated Activity

Chemical extractability before and after incubation with earthworms was assessed after each aging timepoint. Triplicate soil samples (1 g) were transferred to 50 ml Teflon centrifuge tubes and 30 ml of 50 mM hydroxypropyl-β-cyclodextrin (HPCD) (Sigma-Aldrich Co. Ltd., UK) was added to each tube. Furthermore, triplicate soil samples (1 g) were mixed with 5 g of anhydrous sodium sulphate (Fisher) and ground to a fine powder using a pestle and mortar, before being added to 50 ml Teflon centrifuge tubes and 20 ml of dichloromethane (Fisher) was added. The tubes were then placed on an orbital flatbed shaker (Janke and Kundel, IKA-Labortechnik KS 201) at 100 rpm for 24 h. After shaking, the tubes were centrifuged for 1 h at 3000×*g* (Beckman Centaur 2), and 3 ml of supernatant was transferred into a 20 ml glass vial containing 15 ml of Ultima Gold XR scintillation fluid (PerkinElmer, UK). The samples were then stored for 24 h in darkness, and the activity of the supernatant was assessed by liquid scintillation counting (LSC; Canberra Packard Tri-Carb® 2300TR liquid scintillation counter). The extractability was calculated relative to the total ^{14}C-activity in soils at day 0 and compared for aging time and concentration. Residual ^{14}C-associated activity in the soil before and after incubation with earthworms was assessed by sample oxidation.

3.3 Results and Discussion

Terrestrial species through their intimate contact with a diverse range of environmental compartments (*e.g.*, soil, air, water, food) may be exposed to a complex array of contaminants.[4,27] Although there is an urgent need for appropriate bio-monitoring tools to assess the progress of remediation of contaminated sites (land and groundwater), there are also concerns regarding the difficulties in extrapolating laboratory-derived data to the field.[28]

Table 3.1 Properties of the compounds used in this study.

Common name	Structure	Chemical name	Log K_{ow}	Maximum water solubility (mg L^{-1})
B[*a*]P		Benzo[*a*]pyrene	6.13	1.63×10^{-3}
Cypermethrin			6.6	4.0×10^{-3}
Diazinon				
Isoproturon		3-(4-Isopropylphenyl)-1,1-dimethylurea	2.87	65.0

Hence the identification and characterisation of appropriate sentinel organisms, and a greater understanding of their adaptive responses, whether physiological (acclimation) or genetic, is needed.[4,29] For instance, there might well be a genetic component for arsenic resistance.[30]

As efficient prospectors of soil, within which they account for a significant proportion of the biomass of the biota,[31] earthworms may be suitable bio-indicators of contamination. Four compounds were assessed for their potential to form DNA SSBs, which included three pesticides (cypermethrin, diazinon and isoproturon) and a positive control (B[*a*]P) (Table 3.1). These compounds were chosen as the pesticides are widely used and also have differing physicochemical characteristics, including solubility and log K_{ow}. In addition, B[*a*]P was chosen to provide a positive control based on previous work in our laboratory.[17]

The 'comet assay' is a short-term genotoxicity technique that has been shown to be a sensitive method for the evaluation of DNA damage to individual cell genomes. The technique is so named as during electrophoresis in alkali pH media, damaged DNA migrates toward the anode, with greater DNA damage reflected by more migration (Figure 3.3). The result when stained is a bright comet head consisting of nuclear DNA and an extended 'tail', which will vary in intensity and length depending upon the amount of DNA damage. Figure 3.3 illustrates this; in Figure 3.3A one sees the rounded appearance of an undamaged coelomocyte nucleus in comparison to one harvested from an earthworm exposed to cypermethrin for 24 h, where extensive DNA migration is noted (Figure 3.3B). Earthworm coelomic leukocytes (coelomocytes) isolated from earthworms exposed to soils that had been amended with contaminants have been previously demonstrated to be a suitable source of cells for use in comet assay experiments.[22]

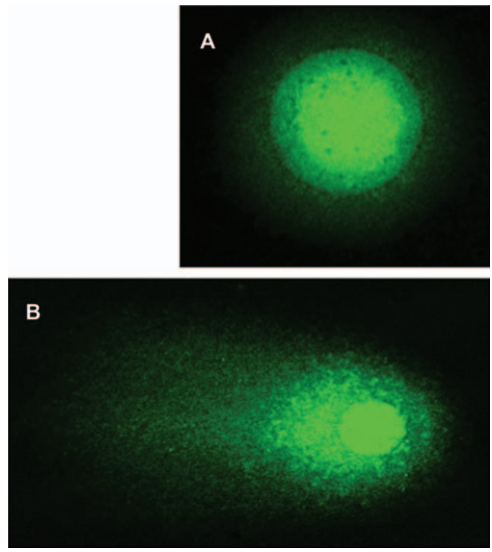


Figure 3.3 Representative photomicrographs of isolated nuclei post-incorporation in the alkaline comet assay. Coelomyocytes harvested from *Aporrectodea longa* following 24 h exposure in soil: (A) Nucleus exhibiting absence of comet formation and (B) nucleus exhibiting comet formation. Images were generated using a Leica TCS SP2 confocal system (Leica Microsystems, Germany) equipped with a DMIRE2 microscope, ×40 objective lens (NA 1.25) and 488 nm argon laser line. Detection was acquired *via* an internal photomultiplier tube (PMT) over the range 624–707 nm for PI (to stain nuclei).

3.3.1 Comet Generation from Differing Compounds

It has been demonstrated previously that exposure to cypermethrin resulted in significant comet production in a number of different systems, including two cell types isolated from the insect *Drosophila melanogaster*.[32] However, as yet there has been little work done on the genotoxicity of these compounds *in vivo*, and particularly in an environmentally relevant exposure setting. The potential of the different compounds to generate DNA SSBs in earthworms was assessed by exposure for 24 h to soils that had been amended with the pesticides and then allowed to 'age' for 24 h (Figure 3.4). Three pesticides were used in this assay, and were applied to the soil at 0.1, 1.0 or 10.0 times the recommended field application rate. This corresponded to concentrations of cypermethrin of 0.08, 0.8 or 8.0 mg kg^{-1}, of diazinon of 0.3, 3.0 or 30.0 mg kg^{-1} and of isoproturon of 0.28, 2.8 and 28 mg kg^{-1}. In addition, B[*a*]P was also investigated as a positive control using concentrations of 0.1, 1.0 or 10 mg kg^{-1}, which had been tested previously in this system (Figure 3.4).

Soils were amended and sterilised. This was considered important as it removed any biological 'influence' on the soil aging process. There may be some potential for exposure to sterile soil having a negative impact upon the

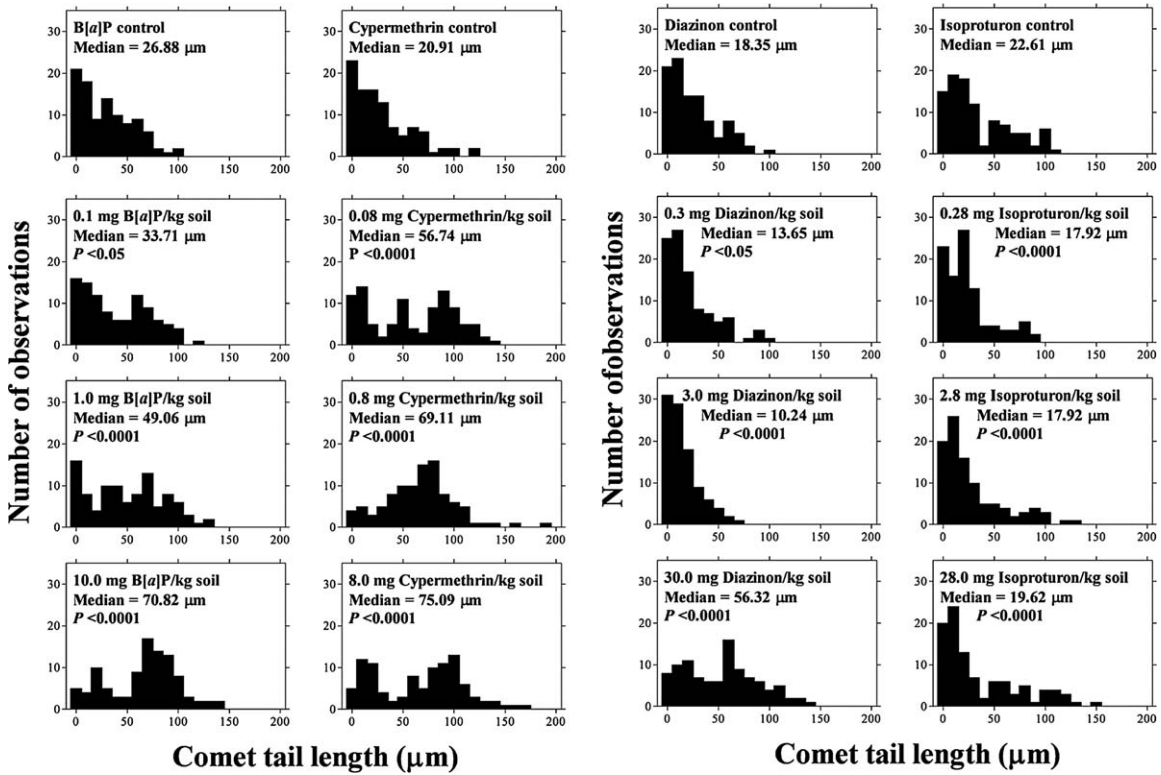


Figure 3.4 Distribution of comet tail length (CTL) in coelomocytes harvested from *Aporrectodea longa* following 24 h exposure in soils that had been amended with ^{12}C compounds (B[*a*]P, cypermethrin, diazinon or isoproturon). Earthworm coelomic fluid containing isolated coelomic leucocytes (coelomocytes) was collected using a sonication method. Following incorporation into the alkaline comet assay, a total of 100 images per treatment were analysed, which consisted of 50 each from duplicate slides produced from two specimens. The vertical axis represents the number of observations and the horizontal axis represents CTL in µm. Median CTL for each treatment condition is shown in each panel. Increases were assessed for significance with the corresponding control using the Mann–Whitney test.

health and activity of the earthworm specimens employed in this study. However, there was no evidence of an impact upon the formation of comets in all the controls produced in this study. In specimens incubated in soils amended with B[*a*]P or cypermethrin, significant dose-related increases in DNA SSBs are observed. B[*a*]P, a known genotoxin, induces a clear dose-related increase in comet formation. Likewise, cypermethrin induces a similar dose-related increase in comet formation with significant ($P<0.0001$) elevations in levels of DNA SSBs observed even at the lowest exposure levels. In contrast, only the highest exposure levels of diazinon induces significant ($P<0.0001$) elevations in DNA SSBs whereas isoproturon appears to significantly ($P<0.0001$) reduce comet-forming activity.

Cypermethrin is a synthetic pyrethroid insecticide that has been shown to induce comet-forming activity in earthworms,[34] which may be associated with oxidative stress.[35] In this context, even at the lowest concentration tested a significant ($P<0.0001$) elevation in comet-forming activity was observed. Diazinon is a thiophosphoric acid ester that is used as a non-systemic organophosphate insecticide. Although previous *in vitro* studies have shown it to induce elevations in micronuclei at low-dose levels,[33] herein significant increases in DNA SSBs are only noted following the highest exposure level. Of course, effects in coelomocytes can only be taken as a surrogate marker of systemic alterations in this *in vivo* model. It remains to be ascertained whether induction of DNA SSBs in coelomocytes reflects site-specific alterations in other tissues (*e.g.*, in the gastrointestinal tract, gonads, *etc*.) where physiological factors such as hormone responsiveness, metabolic turnover or cell proliferation might differ. Isoproturon is used as a herbicide that can enter the *in vivo* organism either through inhalation, ingestion or dermal absorption. Previously, it was shown not to be comet-forming.[37] Likewise, in this study there is a lack of induction of DNA SSBs following isoproturon exposure. The question then is whether this herbicide is non-genotoxic or whether induction of DNA SSBs measurable in the alkaline comet assay is an appropriate endpoint measurement.

3.3.2 Changes in Comet Formation Following Exposure to Aged Cypermethrin Residues

In order to assess the effect of differing aging time upon the potential for the production of DNA SSBs, earthworms were exposed for 24 h to soils that had been amended with cypermethrin and allowed to 'age' for periods of 24 h, 7 d, 30 d or 100 d. The concentrations of cypermethrin tested were 0, 0.8, 8.0 or 80.0 mg kg^{-1}. The relative susceptibility of coeleomic fluid cells to DNA damage was assessed by comparison to control soils amended with carrier solvent only and the data is presented in Figures 3.5 and 3.6. After an aging period of 24 h there was clear evidence of comet formation. After aging for 7 days, there continued to be a dose-related increase in comet formation. There is a significant ($P>0.05$) reduction in comet formation in the

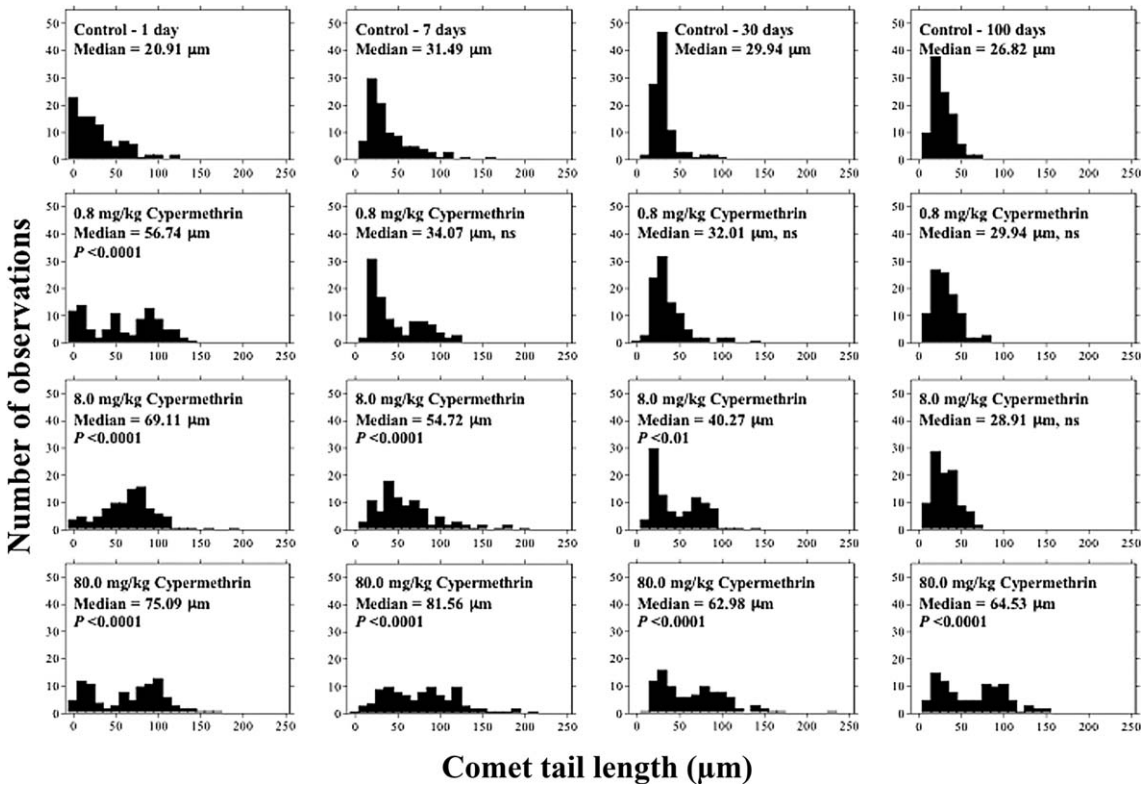


Figure 3.5 Comet-forming activity in coelomocytes harvested from *Aporrectodea longa* following 24 h exposure in variously aged soils. Soils were amended with ^{12}C-cypermethrin only, to a final concentration of 0, 0.8, 8.0 or 80.0 mg kg^{-1}. The soils were stored in the dark at 20 °C, then sampled after 24 h, 7 d, 30 d and 100 d. Then, one earthworm (*A. longa*), which had been depurated for 24 h, was then added to individual glass jars containing the variously aged soils. After 24 h exposure, the earthworm was removed from the soil and coeleomic fluid was collected prior to incorporation into the alkaline comet assay. Following incorporation into the alkaline comet assay, a total of 100 images per treatment were analysed, which consisted of 50 each from duplicate slides produced from two specimens. The vertical axis represents the number of observations and the horizontal axis represents CTL in µm. Median CTL for each treatment condition is shown in each panel. Increases were assessed for significance with the corresponding control using the Mann–Whitney test.

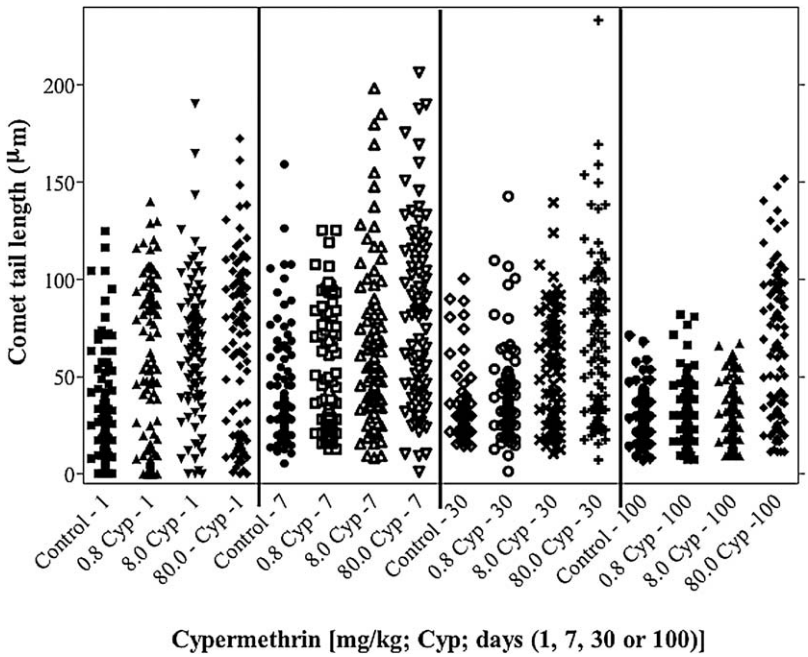


Figure 3.6 Comet assay results presented as scatter plots, which show the levels of DNA damage induction in each individual coelomyocyte nucleus following 24 h exposure in variously aged soils. Soils were amended with ^{12}C-cypermethrin only, to a final concentration of 0, 0.8, 8.0 or 80.0 mg kg^{-1}. The soils were stored in the dark at 20 °C, then sampled after 24 h, 7 d, 30 d and 100 d. Then, one earthworm (*Aporrectodea longa*), which had been depurated for 24 h, was added to individual glass jars containing the variously aged soils. After 24 h exposure, the earthworm was removed from the soil and coeleomic fluid was collected prior to incorporation into the alkaline comet assay. Following incorporation into the alkaline comet assay, a total of 100 images per treatment were analysed, which consisted of 50 each from duplicate slides produced from two specimens. CTL (μm) was used as a measure of DNA damage.

0.8 mg kg^{-1} cypermethrin treatment compared to the corresponding control. However, significant increases in CTL above control levels were observed for the 8.0 ($P<0.001$) and 80.0 ($P<0.001$) mg kg^{-1} treatments. Similarly, after 30 days aging there was a significant increase in comet formation in the 8.0 ($P<0.01$) and 80.0 ($P<0.001$) mg kg^{-1} treatments above control levels. However, there was a decline in median tail length between 7 and 30 days' aging, from 54.72 to 40.27 μm and 81.56 to 62.98 μm in the 8.0 and 80.0 mg kg^{-1} treatments, respectively. After an aging period of 100 days, only the highest concentration treatment of cypermethrin (80.0 mg kg^{-1}) showed a significant ($P<0.001$) increase in comet activity above control levels. These interactions between a pollutant and soil can be termed 'aging', and the retention of a compound within the soil matrix is often referred to as the

Table 3.2 Uptake of differing compounds by *Aporrectodea longa* placed in contaminated soil for 24 h. Total uptake expressed as percentage of total ^{14}C-associated activity recovered.

Compound	Concentration (mg kg^{-1})	%Uptake
B[*a*]P	0.1	0.19 ± 0.02
	1.0	0.27 ± 0.04
	10.0	0.41 ± 0.02
Cypermethrin	0.8	0.68 ± 0.04
	8.0	0.45 ± 0.06
	80.0	0.41 ± 0.01
Diazinon	0.3	0.81 ± 0.14
	3.0	0.84 ± 0.20
	30.0	1.20 ± 0.14
Isoproturon	0.28	0.43 ± 0.11
	2.8	0.64 ± 0.01
	28.0	0.59 ± 0.05

formation of 'bound' residues. Bound residues can be defined as chemicals that remain in the soil or organic matter, even after exhaustive sequential extractions.[36]

3.3.3 Uptake of ^{14}C-Compound

Table 3.2 shows that *A. longa* takes up a very low percentage, irrespective of the concentration added of each of the test agents (B[*a*]P, cypermethrin, diazinon and isoproturon); in general, less than 1%, and this equates to more than a 100-fold lower concentration than that originally added to the soil samples. Irrespective of this, it is clear from the results using the alkaline comet assay that this approach was sufficiently sensitive to identify environmentally relevant real-world and sub-lethal exposures to genotoxic contaminants.

3.4 Conclusion

The results of this study point to a potential sub-lethal biomarker of soil contamination using *A. longa* as a sentinel organism. Future studies will further investigate the reproducibility of these results in comparison with other earthworm tissues and species. Other environmental contaminants require examination using this biomarker model and there is a need to elucidate underlying mechanisms of action. This *in vivo* approach might facilitate the laboratory testing of the bioavailability of contaminants in different soil samples, either spiked or obtained from different sites. Clearly, this approach offers a more sensitive method for determining the bioavailability of contaminants than the putatively less sensitive methods currently used in existing acute and chronic strategies, which measure relatively insensitive end-points, such as respiration, reproduction and mortality.

However, questions relating to the definition of bioavailability and what these less sensitive methods actually measure have been raised recently and have implications for the risk assessment of contaminated land.[38] The results of this study indicate that *A. longa* may be a suitable organism for the investigation of soil contamination by pesticides. Approaches such as the 'comet assay' may be of use in environmental fate and behaviour studies. There is potential that it could be used in a variety of other environmental field contexts, such as nanotoxicology, amphibians or fish.[39–43] It is hoped that this work may assist the development of pesticide management strategies to reduce the risk to non-target receptors. Our ultimate aim is to develop an experimental model that can assess ecotoxicological risk within a regulatory remit.

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CHAPTER 4

Evaluation of the Genotoxic Potential of Contaminated Soil Employing the Snail Helix aspersa

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4.1 Introduction

Large amounts of harmful chemical products are constantly released into the environment as a consequence of population growth factors, such as industrial development, intense urban activities, use of natural resources, mining and agriculture, creating serious environmental problems. Consequently, it is difficult to monitor the types, quantities and effects of toxic substances that are entering the terrestrial environment owing to: (1) the complexity and cost resulting from the identification of the chemical substances involved; and (2) the limited environmental application of traditional chemical analyses that do not reflect the effects on the organisms or inform about the possible

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interactions between the substances (additive, antagonistic or synergistic), as well as their bioavailability.[1,2]

The scientific community has emphasized the need to link chemical analyses with biological responses obtained from the use of bioindicator organisms in order to provide a better idea of the environmental impact of soil contamination. Bioindicators are species, ecological community, or biological processes that are typically monitored over time for changes (as in abundance or health) and are used to assess the state of a particular environment.[3]

The methods available for environmental risk assessment of potentially contaminated soils present some difficulties, mainly to establish reliable levels of exposure to organisms and to identify representative biological responses in laboratory tests. Thus, invertebrate groups are appropriate as bioindicators or sentinel organisms owing to their close contact with soil,[4] and they can be exposed both *in situ* and under laboratory conditions. Among terrestrial invertebrates, the gastropod *Helix aspersa* has been used for a long time to reveal field contamination.[4,5] The common garden snail *H. aspersa*, syn. *Cantareus aspersus*, is a phytophagous, detritivorous pulmonate mollusk included in various food chains and eaten by humans in some countries.[6] *H. aspersa* is native to Western Europe and was introduced on other continents, mainly New Zealand by France in 1860, remaining one of the most abundant terrestrial mollusks.[7]

The advantages of using this land snail include: (1) easy handling and acclimation in the laboratory; (2) sensitivity and resistance to different assays; and (3) different routes of exposure. De Vaufleury[6] stated that while being exposed to contaminants either by the plants they eat or by air they breathe, snails can also be contaminated by the soil through digestive and cutaneous routes.

Furthermore, in land snail *H. aspersa* it is possible to observe alterations at the molecular or biochemical level promoted by the presence of pollutants in the soil. Nascimento *et al.*[8] showed that techniques with responses at lower levels of biological organization are considered more preventive than responses at more complex levels, such as population and ecosystem levels, because when a significant alteration is evident, the ecosystem is severely impacted.

H. aspersa have the capability to accumulate different classes of chemicals and are useful species for monitoring metals and pesticides,[9,10] as well as plant extracts, contaminated or not.[11] Xenobiotics accumulated through different routes are transported by blood cells to the digestive gland, which is also the main target organ for metabolic and detoxification processes.[10] Thus, in the last few years, *H. aspersa* have been used in environmental monitoring of enzymatic biomarkers since the increase or inhibition of the activity of certain enzymes can explain a possible response to environmental stress. However, owing to the importance of ensuring the genetic integrity of the organisms, genotoxicity biomarkers are gaining attention in the evaluation of the toxic potential of contaminated samples in landsnails.

DNA alterations induced by chemical and physical pollutants include single and double strand breakages, induced directly or indirectly by an

interaction with oxygen radicals, DNA–DNA crosslinks, and DNA–protein crosslinks.[12] Different methods have been established to evaluate DNA alterations, however the Comet assay and the Micronucleus test are the most often applied techniques in *H. aspersa*. The Comet assay or single-cell gel electrophoresis (SCGE) is a rapid and sensitive technique that detects DNA strand breaks, measuring the migration of DNA from immobilized individual cell nuclei.[13] The Micronucleus assay has become one of the most widely used methods for measuring structural and numerical chromosomal changes in different systems and detecting cytoplasmic masses of chromatin, which are not integrated in the daughter nuclei during mitosis and which remain in the cytoplasm after cell division, called micronuclei.[12]

Therefore, the aim of the present chapter was to compile and discuss information present in the literature about the use of *H. aspersa* in the analysis of contaminated soil with diferent agents, considering genotoxicity.

4.2 The Major Groups of Soil Contaminants

Natural soil is one of the key elements enabling life on earth. It plays a central role in all terrestrial systems, provides a habitat for microorganisms, plants and animals, and acts as a water filter and storage area. Pollutant dynamics and bioavailability differ greatly in soil systems according to its characteristics, if arable or mineral or grassland, *etc*. Soils receive contaminants from a wide range of sources, including: fossil fuel combustion, automobile exhaust, metal-working industries, chemical industries, waste disposal by incineration, agricultural chemicals, and domestic and industrial waste disposal,[14] that can be leached into groundwater, rivers and lakes. Thus, the soil contamination can include a complex mixture of chemicals compounds that cause serious problems to the environment and to organisms that living there. First the characteristics of some major groups of land contaminants will be discussed.

4.2.1 Heavy Metal

Heavy metal contamination refers to the excessive deposition of toxic heavy metals in the soil caused by human activities, which have gradually increased in recent years. The main anthropogenic sources of metals include deposition in the atmosphere, mainly of gas and dust produced by energy, transport, metallurgy and production of construction materials, sanitary sewage, chemical wastewater, mining and industrial solid waste, and the use of pesticides and fertilizers.[15]

Heavy metals in the soil include some significant biotoxic metals, such as mercury (Hg), cadmium (Cd), lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), copper (Cu), nickel (Ni), tin (Sn), and vanadium (V).[15] Furthermore, the mobility and bioavailability of metals depend on the soil conditions, but some, such as Cd and Zn, which tend to be less strongly sorbed than Pb and Cu, can be leached down soil profiles, especially under acid conditions.[14]

Metal contamination results in serious environmental deterioration because: (1) they are highly persistent in the soil;[16] (2) they can express their pollutant potential directly in soil organisms by availability to plants and transference to the food chain, both by plants and by the contamination of superficial or groundwater;[17] (3) they are colorless and odorless, so they are difficult to notice (they are difficult to find); (4) once the soil is heavily contaminated by metals it is difficult to perform remediation; and (5) it occurs by complex mixture, which will always amplify the contamination.[15]

It is well known that metals have the potential to cause harmful effects on human health and on the environment when imbalanced. In general, genotoxicity of inorganic elements is caused by indirect mechanisms, a major one of which involves interference with cellular redox regulation and the induction of oxidative stress (ROS generation), which may cause oxidative DNA damage.[18,19]

4.2.2 Organic Contaminants

More than 20 000 organic contaminants are already known, and this number will increase as analytical methods are further refined and more studies are made of materials containing complex mixtures of organic pollutants.[14] Some of the main pollutants that cause concern in relation to soil contamination are polycyclic aromatic hydrocarbons (PAHs),[20] which are very important persistent organic pollutants in the environment.[14] PAHs are produced by many sources but many industrial processes, such as oil refining, incomplete combustion of organic materials and coal-burning, can be highlighted.[21]

Soil receives considerable amounts of PAHs, which enhances the probability of exposure of humans and animals to these compounds.[22] PAH can induce DNA lesions as single-strand breaks *via* DNA repair mechanisms,[23,24] electrophilic metabolites that covalently interact with DNA,[25] and can form adducts with purines, especially with guanine, after metabolic activation by the P450 enzymatic complex.[26]

Another potentially pollutant substance is pesticides. Pesticides can be soil contaminants as a result of persistence after use on crops, runoff from treated land, accidental spillages, or pesticide manufacture.[14] These compounds are among more than 1000 active ingredients that are marketed as insecticides, herbicides, and fungicides.[27] Frequently, less than 10% of the pesticide applied reaches its target; the rest is dispersed in the environment. Humans can be affected through the food chain or in contaminated drinking water, which the pesticides, or their decomposition products, reach either by leaching into ground water or their runoff into surface waters.[14]

There is evidence regarding the association of pesticide exposure with the incidence of chronic diseases and introduction of genetic damage.[27] Pesticides have been considered potential chemical mutagens: experimental data revealed that various agrochemical ingredients possess mutagenic properties. As most environmental contamination by pesticides involves

mixtures, the genotoxic potential evaluated for single compounds could not be extrapolated to organisms exposed.[28]

4.2.3 Sewage Sludge

Sewage sludge is generated in sewage treatment plants. These pollutants contain a wide range of environmental contaminants owing to the diverse sources of effluents discharged into sewers, which include human excretion products, household chemicals, automobile fuels, lubricants and cleaning compounds, stormwater runoff from highways containing PAHs and other fuel combustion products, metals and effluents from many different industries.[14]

In its composition, sewage sludge can contain metals, organic chemical compounds (halogenated aromatics, polychlorinated biphenyls, polychlorinated terphenyls, polychlorinated naphthalenes, polychlorobenzenes, aromatic amines and nitrosamines, phenols and halogenated aromatics containing oxygen, PAHs, phthalate esters and pesticides) and biological elements (pathogens) that, in contact with man and/or fauna and flora, may cause contamination and diseases.[14,29]

Based on a series of experiments on genotoxic/mutagenic and carcinogenic elements, Silva *et al.*[30] stated that sewage sludge does not induce genotoxicity and carcinogenesis. However, Martins *et al.*[31] showed that sewage sludge can induce different classes of genetic damage, such as gene mutations, chromosomal aberrations, and micronuclei in agricultural plants, and that crops grown on excessively high doses of sludge can bioaccumulate toxic compounds in their tissues, and these can be transferred to potential consumers.

4.3 *Helix aspersa* for Biomonitoring of Contaminated Soil

The high quantity of contaminants in a specific soil type can, depending on the proportion of quantity and quality, change the natural characteristics of the environment. As previously presented, many soil pollutants are produced and can change the way soil is used and have negative effects on environmental health.[32,33] Therefore, specific approaches and models are needed to assess the impact of soil contamination on terrestrial ecosystems.

Thus, these biological models should be able to show differences between natural oscillation changes, seasonal rain and drought cycles and anthropic stressors.[34] Some researchers agree that the use of the land snail known as *H. aspersa* (Mollusca: Gastropoda: Stylommatophora: Helicidae) to biomonitor environmental contamination is one of the best options because it is easy to acclimate and manipulate.[7,35,36] The main biological characteristics of this mollusk are: gray, damp skin; four tentacles in the anterior portion, two small ones to eat and two big ones containing the eye structure;

the shell is light brown with dark brown in some spheres delimiting the spiral, but color is variable; it presents a big and spherical slim superficies, shiny and without perforations, with wrinkles delimiting the shape.[37] They move using a muscular organ that drags their body. In the foot, they have a hidden gland that secretes almost gray mucus, which leaves a trail when the body is in locomotion. Members of this species are most active during the night, because of humidity, but during the day can be observed after rain. They are most active at temperatures between 4.5 and 31.5 °C. Their development cycle is considered short with stages from egg to adult life lasting on average 60 days, and occurs throughout the year. Adults have a 28 to 32 mm diameter shell. They are incomplete hermaphrodites, with both female and male organs; nevertheless they need to join to reproduce. All of them are able to lay eggs.[37]

The common garden snail *H. aspersa* is one of the sentinel species proposed to map the environmental concentrations mainly of toxic metals, using either eggs or some specific body tissue. Many studies have used mollusks as an indicator of environmental pollution.[6] When a brief review of the use of *H. aspersa* for contaminated soil biomonitoring was performed, 69 articles were found. Table 4.1 summarizes some of these studies, limited to those that include organisms directly exposed to contaminated soil, both *in situ* or in laboratory conditions.

Although there is a 1973 publication with *H. aspersa*, it was only from the middle of the 1990s that the use of the gastropod as an environmental pollution bioindicator really began. Almost all studies involve contamination by heavy metals, such as Cd, Ni, Cu, Pb and Zn,[10,38–45] but other contaminations were also demonstrated, such as pesticides and herbicides,[44,69] *Bacillus thuringiensis* insecticidal Cry1ab toxin,[54] and coal.[47] The researchers carried out tests in general with a focus on identifying bioaccumulation of heavy metals and their concentrations in different snail tissues (in general using the entire body, including viscera and foot), employing techniques such as graphite furnace atomic absorption spectrophotometry.

Other evaluations, such as PAH using HPLC, and the Comet assay and the Micronucleus test as genotoxic biomarkers, were also found in the literature. According to some authors, the most suitable tissues for analysis involving the bioaccumulation of heavy metals are the foot and visceral mass.[33,49,74] Viscera contents of *H. aspersa* include tissues of the digestive, circulatory, excretory, respiratory and reproductive systems.[48] All these studies have demonstrated the efficiency of the land snail as a biomonitor because they are capable of absorbing contaminants from different sources in various tissues, such as the hemolymph, shell, stomach, lung and foot. Tests using eggs are very interesting because they allow investigation of individuals from their contaminated habitats or exposed to contaminated soil in the laboratory, and the maintenance of organisms in the laboratory until reproduction. The eggs generated in the laboratory are analyzed together with adult individuals.[39] Different studies demonstrated that eggs can present metals transferred by their parents as a consequence of previous exposure.[10,54,61,66,68,74]

Table 4.1 Assessment of soil contamination using *Helix aspersa*.

Origin of soil	Contaminants	Period of exposure	Parameter evaluated	Body part and/or eggs	Ref.
Radiation zone	Cs-137, ^{226}Ra	24 days	Bioaccumulation	Viscera and foot	Gaso *et al.*[38]
Urban zone	Heavy metal exposure	4 months	Bioaccumulation of heavy metals	Eggs	Beeby and Richmond[39]
		7 days		Hepatopancreas	Beeby and Richmond[40]
	Pb	7 days	Bioaccumulation of Pb	Viscera and foot	Beeby and Richmond[41]
	Zn, Cu, Cd, Pb	*In situ,* all life	Accumulation of metal	Viscera	Notten *et al.*[42]
Industrial zone	Cd, Pb, Zn, Mn, Fe	84 days	Bioaccumulation of metals	Viscera and foot	Gimbert *et al.*[43]
		14 days		Eggs	Druart *et al.*[44]
		2 months		Viscera and foot	Coeurdassier *et al.*[45]
		6 months		Head and hepatopancreas	Larba *et al.*[46]
Coal mining zone	Coal, metals	5 and 7 days	Bioaccumulation of metals	Foot and hemolymph	Souza *et al.*[47]
Soil from regions contaminated with heavy metals	Cd, Cu, Ni, Pb, Zn	28 days	Bioavailability of metals in the environment and bioaccumulation of metals	Viscera and foot	Vaufleury *et al.*[48]
		5 and 7 weeks		Viscera and foot	Scheifler *et al.*[49]
		56 days		Viscera and foot	Scheifler *et al.*[50]
		7 weeks		Viscera and foot	Viard *et al.*[51]
		3 months		Viscera	Vaufleury *et al.*[52]
		In situ, all life		Viscera	Mulvey *et al.*[53]

	Toxin (*Bacillus thuringiensis* insecticidal Cry1ab)	47 weeks	Bioaccumulation of toxin	Eggs	Kramarz *et al.*[54]
	Cd, Pb and Zn	28 days	Bioavailability of metals	Viscera	Pauget *et al.*[55]
	Cr	28 days	Accumulation of metal in plants	Viscera	Eybe *et al.*[56]
	Cr and Pd	28 days	Bioavailability of metals	Viscera	Pauget *et al.*[57]
	Zn and Cd	4 and 6 weeks	Accumulation of metal in plants	Viscera and foot	Sinnett *et al.*[58]
	Cd, Pb and Zn	28 days	Accumulation of metal in plants	Viscera	Fritsch *et al.*[59]
Artificially contaminated	Cd, Cs-137, Ar, Zn, Cu	84 days	Bioaccumulation of metals	Viscera and foot	Gimbert *et al.*[60]
		28 days		Foot	Kramarz *et al.*[61]
		2 weeks		Viscera and foot	Fritsch *et al.*[62]
		28 days		Viscera and foot	Bourioung *et al.*[63]
		4 weeks		Viscera and foot	Cœurdassier.[64]
	Cd, Cr , Pb, Zn	28 days	Bioavailability of heavy metals	Viscera and foot	Vaufleury *et al.*[65]
	$CaCO_3$	66 days	Bioaccumulation of $CaCO_3$	Eggs	Crowell *et al.*[66]
	Mg	28 days	Accumulation of metal	Viscera	Gimbert *et al.*[67]
	Herbicides	168 days	Bioaccumulation of herbicides	Viscera	Druart *et al.*[68]
	Insecticides	28 days	Bioaccumulation of insecticides	Viscera	Hartnik *et al.*[69]
	Organophosphorus pesticide	4 weeks	Bioaccumulation of pesticides	Viscera	Coeurdassier *et al.*[70]
Agricultural soil, artificially contaminated	Heavy metal exposure Zn, Cu, Cd	2 weeks	Accumulation of metal	Viscera	Coeurdassier *et al.*[71]
	Cd	28 days	Cd	35 Viscera	Li *et al.*[72]
	Polycyclic aromatic	2 and 4 weeks	Bioavailability of Polycyclic aromatic	32 Viscera	Sverdrup *et al.*[73]

Risk assessment of field soil, contaminated soil, and soil material cannot be based only on the chemical–analytical determinations of the total contents of specific contaminants because they do not provide information on unexpected substances, metabolites of pollutants, and bioavailable fractions. Therefore, the chemical–analytical determinations of the total pollutant content are not sufficient for the qualitative and quantitative detection of potential health risks. For an appropriate consideration of all these factors, genotoxicity tests using biomonitors have to be applied, which reflect the biological effect of the soil pollutant or of miscellaneous exposure.

4.4 Genotoxicity Tests with *H. aspersa* and Contribution to Environmental Research

Exposure to toxic agents can lead to a variety of effects on living organisms, which can present immediately or over the long term, such as DNA damage and diseases that include cancer and developmental disorders, among others.[75] Agents that have this ability to change the DNA in form, structure or sequence are called genotoxic agents. Genotoxicity tests have been used as a promising alternative to indicate soil quality, and they act as a predictor of early damage to the genetic content of living beings.[76] Thus, the assessment of genotoxic risk in soil samples using genotoxicity biomarkers allows us to understand the processes involved and the interaction of these substances with the cell, mimicking the possible genotoxic effects on organisms, including humans.

The analysis of DNA alterations using *H. aspersa* has been shown to be a highly suitable method for evaluating the genotoxic contamination of soils, being able to detect exposure to low concentrations of contaminants. In general, genotoxicity assays have the advantage of detecting and quantifying the impact of DNA damage without requiring detailed knowledge of the specific agent present in the contaminated site. Contaminants in the environment, including genotoxic agents, could occur as complex mixtures, and the risks of such mixtures, the presence and potential interaction of unknown substances, could lead to a discrepancy between the present and the predicted risk. Therefore, mollusks have been used to evaluate soils with different compositions, either at the contaminated site or with samples from the site under laboratory conditions.

Genotoxicity studies using *H. aspersa* began after 2005 (Table 4.2), and have presented a very good response to metals and PAHs. All studies on DNA damage using *H. aspersa* are summarized in Table 4.2. Genotoxic effects in these studies were evaluated by Micronucleus (MN) test and the Comet assay, and were also associated with other tests to help answer questions concerning the mechanisms of genotoxicity, such as oxidative stress and chemical element quantification. When these methods are performed in the laboratory or even *in situ*, they do not reproduce all the complexity of what happens in nature, but they do give short-term indications of the impact of the contaminants.

Table 4.2 Evaluation of genotoxicity using *Helix aspersa*.

Exposure	Agents	Parameters studied	Exposure time	Results	Ref.
Coal tailings	Inorganic elements and PAHs	Comet assay, MN test, oxidative stress	24 h, 5 and 7 days	DNA damage in hemolymph cells for all periods (Comet assay) and oxidative stress; relation with inorganic elements and PAHs	Souza *et al.*[47]
Coal tailing and lettuce from miner's garden	Inorganic elements; pyrite tailings	Comet assay	6, 12, 24, 48 h, 7 days	DNA damage in hemolymph cells for 24 h, 48 h, and 7 days exposure periods; relation with inorganic elements	Leffa *et al.*[80]
Tobacco leaves	Tobacco composition and inorganic elements	Comet assay, MN test, oxidative stress	24, 48, 72 h	DNA damage (Comet assay and MN test) and oxidative stress in hemolymph cells for all exposure periods; relation with nicotine, coumarins, saponins, flavonoids and inorganic elements	Da Silva *et al.*[36]
Antarctic plant species	Plants composition	Comet assay	7 days	DNA damage in hemolymph cells related with only one plant sample; relation with plant composition	Pereira *et al.*[11]
Urban air pollutants	Particulate matter	Comet assay	7, 15, 30 days	DNA damage in hemolymph and lung cells for all exposure periods (lung > hemolymph) (30 > 15 > 7 days)	Da Silva *et al.*[35]
Urban area with heavy traffic: PAHs	Particulate matter	Comet assay	15 days	DNA damage in hemolymph cells; relation with traffic and PAHs	Ianistcki *et al.*[84]
Coal dust and by-products from burning coal	Heavy metals	Comet assay	*In situ*, all life	DNA damage in hemolymph cells for snails exposed to the sites near the power plant	Angeletti *et al.*[85]
Electromagnetic field	Electromagnetic low frequency	Comet assay, oxidative stress	10 days	DNA damage and oxidative stress in digestive glands and hemolymph cells	Regoli *et al.*[86]
Cd	Cd solutions	Embryotoxicity; RAPD	24 h	Embryotoxicity of Cd associated with genomic and genotoxic effects.	Baurand *et al.*[87]

Since there are few studies using genotoxicity and *H. aspersa*, their results deserve to be highlighted. Souza *et al.*[47] used *H. aspersa* in dermal contact with soil samples from different sites in Charqueadas (RS, Brazil) to evaluate the genotoxic potential of soil contaminated with mineral coal. Thirty terrestrial snails were exposed to different treatments: 20 were exposed to the soil from two different sites at Charqueadas and 10 were non-exposed to the soil. Comet assay, Micronucleus test and oxidative stress tests were performed. Furthermore, this study quantified the inorganic elements present in soil samples by PIXE technique and PAH by HPLC. This evaluation shows that, in general, soils from the Charqueadas sites demonstrated a genotoxic effect associated with increased oxidative stress, and inorganic and PAH content. These results demonstrate that the coal pyrite tailings from Charqueadas are potentially genotoxic.

Plants can bioaccumulate inorganic elements. This property is influenced by the physicochemical characteristics of soil, plants, and the exposure time range of dispersion.[77–79] Another study using *H. aspersa* exposed to coal was carried out by Leffa *et al.*,[80] but in this case it assessed the genotoxic potential of mineral coal tailings and lettuce grown in a coal tailings deposit. Animals were divided into three groups, clustered in Plexiglas cages: control (animals fed with organic lettuce), coal tailings (animals living in a layer of pyrite tailings and fed with organic lettuce), and mine lettuce (animals fed with lettuce grown in an area located in a coal tailings deposit). Results showed that the animals in the coal tailings and mine lettuce groups presented higher levels of DNA damage compared to the control group at all exposure times, but with the peak of DNA damage at 48 h and 96 h. These results demonstrate that the coal pyrite tailings are potentially genotoxic.

Da Silva *et al.*[36] aimed to evaluate the genotoxic and mutagenic effects of tobacco leaves, with and without exposure to flumetralin in *H. aspersa,* through dermal exposure. Biomonitoring tests for genotoxic effects were carried out in the form of the Comet assay and Micronuclei test. Chemical element quantification by PIXE and cytochrome P450 quantification were also performed. Results demonstrate that tobacco leaves are genotoxic in *H. aspersa*. The genotoxicity, mutagenicity and enzymatic inhibition caused by exposure to tobacco leaves was probably mediated by the complex mixture of substances (nicotine, coumarins, traces of saponins, flavonoids and different inorganic elements) present in these leaves.

Pereira *et al.*[11] used *H. aspersa* and the Comet assay to investigate the photoprotective effect of the methanolic extracts of three Antarctic plant species (*Deschampsia antarctica* Desv., *Colobanthus quitensis* (Kunth) Bartl., and *Polytrichum juniperinum* Hedw.) against UV-induced DNA damage. Animals were kept in cages and seven animals were considered for each group. The control group was fed only with lettuce, whereas the test groups were fed with the species of plant under study during the 7 days and the Comet assay was performed. In order to evaluate the potential photoprotective effect, hemolymph cells were exposed to UVC radiation at 4.5 $J\,m^{-2}$ and the Comet assay was performed as described by Silva *et al.*[81] and

Hartmann *et al.*[82] with some modifications. In conclusion, the hemolymph cells of animals fed with *D. antarctica* and *P. juniperinum* did not present a significant difference in DNA damage compared to the control group; however, animals fed with *C. quitensis* showed significant DNA damage, suggesting the presence of genotoxins. Cells exposed to UVC showed significantly higher DNA damage as compared to the control. In the groups fed with either *Deschampsia* or *Polytricum* a reduction in DNA damage parameters was observed, suggesting that the treatment was able to protect against UVC-induced damage, which could be attributed to molecules such as flavonoids and carotenoids, which act as UV-sorbing molecules and antioxidants, and also stimulate DNA repair processes.

Soil reflects the level and the spatial distribution of air pollutants such as PAHs.[83] Silva *et al.*[35] verified the feasibility of working with *H. aspersa* in assessing the genotoxicity of atmospheric pollutants and concluded that the mollusk is sensitive as a biomonitor. An increasing number of studies indicate that particulate matter (PM) air pollution can have a severe effect on human health. Ianistcki *et al.*[84] aimed to biomonitor metropolitan areas of Porto Alegre (RS, Brazil) for PAHs associated with atmospheric particles and check their effects on the DNA of the bioindicator *H. aspersa*. The sampling sites are located in the region of Porto Alegre, in an urban area with traffic entering and leaving town. Adult *H. aspersa* land mollusks were acclimatized to laboratory conditions for 7 days, during which time they received *Lactuca sativa* L. leaves from organic cultures and water *ad libitum*. After acclimatization, the snails were clustered and identified as control and test groups. Ten individuals were exposed for 15 days per site. At the end of this period, the hemolymph was sampled. Results show that, in general, the smaller PM size fractions ($PM < 2.5$) have the highest genotoxicity and contain higher concentrations of extractable organic matter. Thus, monitoring of genotoxicity by *H. aspersa* has proven to be an effective and sensitive instrument for better risk assessment in relation to particulate exposure. Mollusks were collected by Angeletti *et al.*[85] at five different locations at decreasing distance from the source of pollution, selecting specimens of *H. aspersa* for sampling. The chimney of the power plant was considered the topological cue from which the distance was calculated. The farthest two locations are rural environments assumed to be free from significant pollution.

Pro-oxidant effects of extremely low frequency (ELF) 50 Hz magnetic fields were investigated by Regoli *et al.*[86] in the land snail *H. aspersa* exposed both to short-term laboratory treatments and under field conditions by maintaining the organisms in the proximity of a power line for up to 2 months. Oxidative disbalance was investigated using catalase, glutathione reductase, glutathione S-transferases, and total glutathione levels, and total scavenging capacity toward peroxyl radicals and hydroxyl radicals. Results indicated oxidative damage caused by ELF magnetic fields, mainly for catalase, glutathione reductase, and the overall capability to neutralize peroxyl radicals. After 10 days of laboratory exposure to different intensities of electromagnetic fields, *H. aspersa* showed significant changes in various biological responses.

Catalase activity decreased at the highest exposure intensity (50 AT), whereas a significant inhibition of glutathione reductase was measured in all the experimental conditions. Other parameters of glutathione metabolism, namely the activity of glutathione S-transferases and the levels of total glutathione, did not exhibit significant changes in snails exposed to ELF magnetic fields. DNA damage data showed a linear increase during the first 20 days in organisms translocated at both sites. Snails generally maintained a more elevated degree of DNA damage at 2.88 AT, and in organisms exposed to the lower environmental ELF intensity of 0.75 AT DNA strand breaks decreased to control values after 40 and 60 days.

Cadmium (Cd) can be toxic to terrestrial snails, but few data are available about its genotoxic effects on early life stages (ELS). The aim of the study by Baurand *et al.*[87] was to investigate the genotoxic potential of Cd in embryos of *H. aspersa* using a new approach that couples Random Amplified Polymorphic DNA (RAPD) and a high-resolution capillary electrophoresis system (HRS). Clutches were exposed to Cd solutions (2, 4, and 6 mg L^{-1}) from the beginning of their embryonic development. In addition to a dose-dependent effect of Cd on hatching rate, DNA fragmentation was observed in embryos that were exposed to 6 mg Cd L^{-1}. The analysis of RAPD products with HRS showed differences between the profiles of exposed and non-exposed embryos, starting at 2 mg Cd L^{-1}. In comparison to the profiles of the control samples, all profiles from the exposed snails exhibited an additional 270 bp DNA fragment and lacked a 450 bp DNA fragment. These profile modifications are related to the genotoxic effect of Cd on the ELS of *H. aspersa.* This study demonstrates the efficacy of coupling RAPD and HRS for rapid and efficient screening of the effects of chemicals on DNA.

4.5 Conclusions

Sentinel species present potential applications for monitoring environmental media, identifying new exposures of potential concern as a result of observing changes in biological organisms, and supporting risk assessment at several points in the processes. Although it is unlikely that biomonitoring species data will be used as the sole determining factor in assessing human health risk, it can be used to weigh the evidence of the approach and provide early warning of situations requiring further studies. Furthermore, they can suggest potential causes and effects. *H. aspersa* as observed in this review can be considered a good tool for biomonitoring soil contamination effects (Figure 4.1). The land snail is exposed to different agents, leading to absorption (breath, skin or digestive) and distribution in the mollusk body. These agents are metabolized and can cause toxicity, oxidative stress or DNA damage. Genotoxicity in these organisms can be associated with oxidative stress, as well as with inorganic and organic elements. The use of genotoxicity biomarkers using this species is of potential interest for the assessment of the pollutants impact on animals under field or laboratory conditions. The obtained results may provide baseline data for future human impact

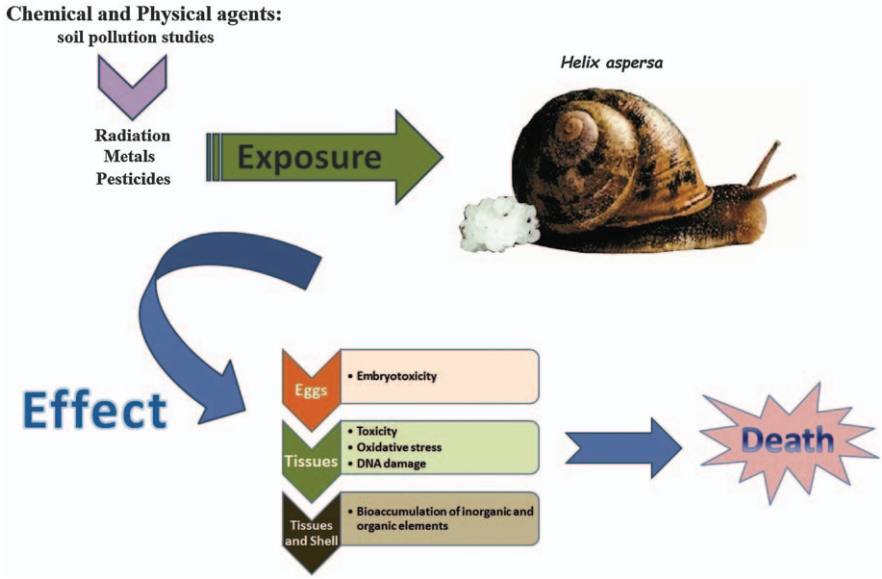

Figure 4.1 *Helix aspersa* exposure to soil contaminants. Snail figure indicates agents of exposure and the effects for which investigations in soil pollution studies were performed.

assessment and risk programs concerning specific pollutants or complex mixture exposures.

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CHAPTER 5

The Use of Spiders in the Assessment of Cellular Effects of Environmental Stressors

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5.1 Introduction

Spiders are commonly found in meadow, forest and agricultural ecosystems. Owing to their high density (even up to 600 individuals per m^2), polyphagy, low nutritional selectivity and high hunting activity, these invertebrates are important regulators of insect population sizes.[1,2] Irrespective of habitat, all spiders hunt for living prey, taking advantage of one of two general hunting strategies, involving web traps or sudden attack. Spiders using the second method are very active and have achieved perfection in direct fighting. The diet of these obligatory predators is dominated by insects belonging to orders such as Diptera, Homoptera, Hymenoptera, Heteroptera, Coleoptera, Lepidoptera, and Collembola, consumed in different proportions, depending on the lifestyle, hunting type and environment of spiders.[3,4] As they occupy different ecological niches in biocoenoses, they can reduce populations of attacked species at various development stages, which makes them effective predators.[5] The role of spiders in the control of insect numbers is particularly important in agrocoenoses, in which their prey include crop pests. Nevertheless, composition of spider diet may be affected by quantitative and

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qualitative changes associated with season, temperature and dynamics of prey populations in the area.

Ecological studies have shown that the structure, size and species composition of spider communities can be correlated with the abundance of other invertebrate groups as well as the state and structure of vegetation; therefore, the discussed predators can serve well as biocoenosis biodiversity indicators.[6–9] Their function should be considered especially for environments in which the natural ecological balance was disturbed by human impact, affecting spiders as well. From the size, species composition, biomass as well as age and sex structure of arachnocoenoses assessed in differently shaped landscapes, including ones affected by intensive agrotechnical measures and industrial pollution, it can be deduced that spiders show species-dependent sensitivity to various forms of anthropopression. As a result, the species composition of spider zoocoenoses is modified and sensitive species are replaced by tolerant ones, whose reproduction is not significantly hindered by the acting environmental stressors.[10–12] It has been documented that environments strongly contaminated with industrial pollution are dominated by spider families less competitive in obtaining food and space, namely the web-building Linyphiidae and non-web-building Lycosidae and Clubionidae.[10,13–17] However, in agrocoenoses treated with synthetic plant protection agents, numbers of Clubionidae spiders were dramatically reduced, while other families were decreased to a lesser degree.[11] Therefore, while some spider groups are able to tolerate chronic contact with pollutants, their survival in such conditions depends on their defense potential. Mechanisms of adaptation/compensation abilities in spiders inhabiting contaminated environments have still not been fully described.

Although the importance of spiders in trophic chains of terrestrial ecosystems seems obvious, only a small amount of research was performed under controlled laboratory conditions in which the levels of selected biochemical parameters and the intensity of stressing factors could be correlated, and the main defense strategies of the investigated predators against different (in terms of quantity and quality) stressors could be studied. Experimental spider colonies are rather rarely maintained due to methodological issues, arising *inter alia* from the requirements of individuals at subsequent development stages. It is very difficult to provide the individuals, particularly young, with optimum conditions, including specific food and humidity requirements. Moreover, irrespective of their age, spiders must be supplied with living prey. Another serious problem in animal maintenance is cannibalism, already frequent at the early stages of the relatively long (as for laboratory animals) ontogenesis and imposing the need to keep individuals separately in laboratory boxes. Web-building spiders must also be provided with spatial conditions appropriate for successful spinning of hunting webs, which, considering the great diversity of their forms and sizes, provides a serious methodological issue. For this reason, tests usually involve non-web-building species, the food, temperature, humidity and light requirements of which are well known and, most importantly, spatial conditions for which can

be provided more easily. Owing to the above-described limitations, most data on the cytotoxic and genotoxic effects of anthropogenic and natural stressors were gathered in research of individuals collected directly from the field or only temporarily kept in the laboratory. Not many studies, particularly of long-term exposure to chemical substances, have been carried out on multi-generation spider colonies.

The following subchapters describe changes that occur in selected biochemical parameters in response to stressing factors affecting spiders living in anthropogenically altered environments, though the predators are not the target of the applied measures and are subjected to their action incidentally. The presented results cover both laboratory tests and measurements taken for individuals collected directly from the field. Such studies may provide a basis for verifying the potential usefulness of the examined parameters as biomarkers of exposure of the investigated animals to factors acting as the broadly understood environmental stress.

5.2 Spiders in Ecosystems Contaminated with Heavy Metals

Effects of environmental pollution are reflected most strongly in predators and parasites, representing the highest trophic levels in food webs of ecosystems and consequently threatened by accumulation of various chemical compounds in tissues. Spiders, being secondary and higher consumers, are therefore potentially more vulnerable to the action of numerous xenobiotics, artificially introduced to the environment, than other organisms. Heavy metals, easily transferred along trophic chains and tending to accumulate at their consecutive levels, seem to impose a particular threat. It is the predatory lifestyle of spiders that brings them into consideration as potential bioindicators of metal contamination in environments. Moreover, these invertebrates, showing high activity and short life span, quickly adjust to environmental changes. Their common and usually abundant occurrence is also worthy of note. In biomonitoring studies, however, seasonal fluctuations in populations of these predators should be considered, as they may account for different metal concentrations recorded in tissues. Sample standardisation, particularly in aspects such as identification of the age and sex of individuals or removal of impurities from the outer body layer, is necessary as well; without it, various methods can provide different results even if applied to the same species. Determining spider species is also a frequent problem for non-arachnologists.

Comparative studies have shown that spiders belong to invertebrates displaying both the highest recorded metal concentrations in bodies and large population sizes in metal-contaminated ecosystems.[18–20] In classification of animals into macro-, micro- and de-concentrators of metals (based on the metal bioconcentration factor in tissues, *i.e.* ratio of a metal's assimilation factor to its elimination factor), spiders are classified as

macroconcentrators.[21] Metal accumulation is facilitated by their polyphagy, food preference towards soft tissues and relatively low metabolic rates in the large midgut glands.[22,23]

Metals are absorbed by spider organisms mainly *via* the digestive tract. Unless removed with faeces, they are usually deposited in midgut gland cells.[24,25] Concentration and duration of storage depends on the type of metal,[26–29] the spider species and its physiological features,[30–36] as well as the sex[33,37] and age of individuals.[38,39] Previous studies have also indicated a relationship between the level of metal accumulation and spider hunting strategy and activity, as well as habitat and type of prey. The feeding strategy determines *inter alia* the probability of direct exposure to potential contamination. Even the straightforward division into web-building and non-web-building spiders points to important differences. Non-web-building spiders have greater contact with polluted soil surface as they constantly touch it, while web-building species usually spin webs on plants, providing some protection from contaminants. Results of comparative studies suggest that in web-building spiders, with a diet dominated by herbivorous insects, concentrations of Cd, Pb, Cu, and Zn are lower than in wandering spiders, which hunt for predatory and detritivorous insects. For example, *Linyphia triangularis* sheet-web spiders (Linyphiidae) (Figure 5.1), despite their low nutritional selectivity and high hunting activity, show a relatively low content of the above-listed elements in their bodies. On the other hand, non-web-building spiders of the Lycosidae family (*Pardosa lugubris, Pardosa palustris, Xerolycosa nemoralis*), irrespective of the level of metal contamination in the environment, display higher concentrations of Cd, Pb, Zn, and Cu in tissues in comparison with values reported for web-building spiders of Araneidae, Linyphiidae and Agelenidae families living in similar environmental conditions.[30,33,37] Other studies also provide evidence for Lycosidae assimilating more Cd and Cu than web-building spiders; however, this fact was not confirmed for Pb.[28]

Figure 5.1 Female *Linyphia triangularis* sheet-web spider (Lynyphiidae) (young individual).

From laboratory experiments on simplified trophic chains using various concentrations of metals administered in prey bodies, it can be concluded that accumulation of cadmium in spiders depends on its content in food and earlier preexposure of individuals to the pollutant. Concentrations of this element analysed in bodies of females and males of a ground-dwelling spider, *P. lugubris*, and its prey, *Drosophila melanogaster*, grown on a control medium supplemented with trace amounts of cadmium or on a medium including the metal in high concentration (426 μg Cd · g^{-1} dry mass), point to the cadmium storage strategy applied at a low content in the diet. However, as the concentration of metal increased in prey bodies, its removal with faeces was intensified.[40] High cadmium assimilation was also demonstrated for *Pirata piraticus* wolf spiders (Lycosidae), fed with fruit flies reared on a medium containing 10 μg g^{-1} Cd during a 42 day experiment. Additionally, the metal was not eliminated from bodies of individuals within a subsequent month of feeding with uncontaminated flies.[29] In other studies, *Agelena labyrinthica* funnel-web spiders (Agelenidae) (Figure 5.2), fed for three weeks with cadmium-burdened *D. melanogaster* (30.0 ± 6.2 μg g^{-1} dry mass of prey), showed several tens-fold higher accumulation of the metal in their bodies in comparison to individuals receiving uncontaminated food. In this investigation it was also noticed that bodies of spiders from heavily polluted areas accumulated more cadmium than at the reference site.[34,39] In *Pardosa piraticus* (Lycosidae), higher Cd, Cu and Zn content was found in spiders from sites with lower total metal content in sediment.[41] Low capacity to store Cd was also reported for females and males of the synanthropic *Steatoda grossa* (Theridiidae) not previously exposed to the contaminant. In bodies of these individuals, fed for four weeks with *D. hydei* flies contaminated with cadmium (48.94 ± 1.78 μg g^{-1} dry mass of prey), concentration of the metal was low, not exceeding 1 μg g^{-1} dry mass in females and attaining a mean of 3.03 μg g^{-1} dry mass in males.[42] These study results, obtained in laboratory conditions, confirmed the importance of physiological and

Figure 5.2 Female *Agelena labyrinthica* funnel-web spider (Agelenidae); (a) leaving its hiding place, (b) entering the funnel-shaped hunting web.

behavioural characteristics in the development of interspecific differences in spiders' ability to bioaccumulate metals.

5.2.1 Cellular Defence Reactions in Spiders from Areas Affected by Industrial Pollution

Effects of stressing factors in organisms are visible primarily at the cellular level as oxidation and denaturation of organic compounds or changes in their synthesis, damage of DNA and biological membranes, as well as disturbance in cell respiration and production of energy. These processes trigger defense reactions, completely or partially restoring cell homeostasis. In extreme cases, at high levels of damage, the cell dies *e.g.* by apoptosis or necrosis. Identification of xenobiotic-induced changes at the cellular level may allow prediction of their adverse effects at higher levels of biological organisation.

5.2.1.1 Non-enzymatic Defence Reactions in Response to Increasing Metal Concentration in Spider Bodies

In invertebrates, metal ions that manage to cross the protective barrier of the intestine can be deposited in mineral granules or bound to low-molecular-weight proteins, metallothioneins (MTs). In spiders, one of the common mechanisms responsible for neutralization of metals is their deposition in midgut glands as intra- and inter-cellular mineral concretions that can be further removed, in a holocrine manner, into the intestine lumen and afterwards excreted.[22,24] Among the four types of granules distinguished, including the intracellular A, B and C and intercellular D, the B type, containing copper, mercury, cadmium, zinc, lead, and iron, seems to play the most important role in metal detoxification in spiders.[43]

This process is also strongly supported by metallothioneins. These low-molecular-weight, cysteine-abundant proteins are of great importance both in the regulation of the concentration of biogenic metals and the inactivation of metals of uncertain biological function. Results of metallothionein content analysis, using the saturation method, in *A. labyrinthica* spiders collected from areas contaminated with heavy metals, point to MT synthesis as a mechanism that significantly contributes to the development of species tolerance to environmental factors.[44] In Lycosids, *Pardosa saltans* and *Pirata subpiraticus*, originating from metal-polluted and unpolluted sites and subjected to controlled laboratory exposure to cadmium, MT synthesis was confirmed to act as an important mechanism enabling survival in ecosystems with metal contamination exceeding certain levels.[45,46]

Spiders' ability to synthesise metallothioneins is species-dependent. In three web-building species, *Araneus diadematus* (Araneidae), *A. labyrinthica* and *L. triangularis*, collected at sites heavily polluted with metals, the highest MT content was measured, with immunoenzymatic assay, in orb-web

spiders, *A. diadematus*. In the bodies of these spiders, metallothionein concentration was positively correlated with levels of Cd, Pb and Zn.[34] Moreover, as indicated by flow cytometry measurements, the number of MT-positive cells in midgut glands of the web-building *A. labyrinthica* and *L. triangularis* and non-web-building *X. nemoralis*, collected at a site strongly contaminated with metals, was sex-specific. The highest percentage of MT-positive cells was recorded in *A. labyrinthica* females.[37] In *X. nemoralis* wolf spiders, both sexes showed similar values of the cells, while in *L. triangularis* their frequency was significantly greater in males than females. Intensified MT synthesis was also reported for young individuals as well as adult females and males of *A. labyrinthica*, fed with *D. melanogaster* grown on a medium supplemented with Cd or Cu. In this spider species, the protective function of MTs could be observed primarily in females; therefore, it can be assumed that in their bodies production of the proteins is an important defense mechanism, triggered in response to increasing metal concentration.[39]

5.2.1.2 *Enzymatic Detoxification*

Among numerous known metabolic reactions in which chemical compounds are processed in organisms, many are typical to both vertebrates and invertebrates. Generally, in biotransformation processes lipophylic compounds are converted to polar products, which usually involves two phases. In the first phase, covering reactions of oxidation, reduction and hydrolysis, the chemical structure of the original substance is modified to intermediate compounds that can be, in the second phase, coupled with various endogenous components, such as glutathione, and in this form excreted from the organism more easily. The most important cytoplasmic enzymes responsible for detoxification of numerous endo- and exo-genous substrates include carboxylesterases (CarE) and glutathione-*S*-transferases (GST). They also play an important role in neutralization of many environmental toxins in spiders. However, according to the results of former research, in these predators enzymatic detoxification of xenobiotics is species- and sex-specific, which can be observed as various biotransformation rates and qualitative differences in metabolic processes modifying the toxic compounds. In conditions of strong environmental contamination with heavy metals, the actively hunting *P. palustris* wolf spiders displayed both enhanced hydrolysis, catalysed by CarE, an enzyme characteristic for the first detoxification phase, and intensified GST-involving coupling reactions, important in the second phase of enzymatic xenobiotic biotransformation. The web-building *L. triangularis*, *A. diadematus* and *Metellina segmentata* (Tetragnathidae) (Figure 5.3) showed greater intensity of GST-catalysed reactions, but lower or unchanged activity of CarE.[30,47] The importance of CarE in detoxifying reactions was also confirmed for *P. lugubris* and *A. labyrinthica* inhabiting forest and meadow ecosystems along a metal contamination gradient. In both species, high metal content in bodies was accompanied by high CarE activity. Additionally, CarE values were greater in females than males, indicating that in female

Figure 5.3 Female *Metellina segmentata* orb-weaving spider (Tetragnathidae) in the centre of hunting web.

individuals, hydrolysis plays a particularly important role in detoxification processes.[48]

All living organisms take advantage of enzymatic and non-enzymatic systems neutralising the reactive oxygen species (ROS), produced in both regular oxidative processes of cells and extracellular fluids as well as during biotransformation of toxic substances entering the body. In spiders from areas contaminated with heavy metals, particular enzymatic antioxidative reactions are intensified as well.[30,49] *P. lugubris* wandering spiders originating from areas heavily polluted with zinc and lead showed high activity of selenium-dependent glutathione peroxidase (GPOX) and selenium-independent glutathione peroxidase (GSTPx), enzymes neutralising hydrogen peroxide and organic peroxides, respectively. In the same conditions, *A. labyrinthica* displayed activity of both peroxidases (GPx) lower than in non-web-building spiders and remaining at similar levels irrespective of site. However, both species differed in glutathione (GSH) concentrations, which were greater in agelenid spiders than in the actively hunting ones.[49] In studies of another lycosid species, *P. palustris*, a correlation was established between a high content of Zn, Cu, Pb and Cd and high catalase (CAT) activity in the bodies of individuals. In the case of *P. subpiraticus*, also representing the Lycosidae family, collected at metal-contaminated sites and under laboratory conditions fed with cadmium-contaminated prey, a gradual decrease was recorded for superoxide dismutase (SOD), CAT and GST activity.[46] Enhanced antioxidative SOD- and CAT-catalysed reactions were also noted in the web-building *L. triangularis*, though their bodies were typified by low metal content.[30] The above-described studies show that even closely related species can react differently to oxidative stress caused by industrial pollution.

Antioxidative systems are found in all cells; however, they are particularly intensive in organs widely exposed to toxic substances, and in spiders are undoubtedly exemplified by the digestive tract, being the main toxin uptake route, and the associated midgut gland, responsible for digestion, storage and detoxification of numerous chemical compounds. Relationships between the level of heavy metals in this organ and the activity of selected antioxidative enzymes were confirmed in studies of *X. nemoralis* spiders. In the species, high concentrations of Cd, Pb, Cu, and Zn in the midgut glands of females corresponded to high GPOX and GSTPx activity and relatively low, as compared to males, CAT activity. Similar analyses carried out for midgut glands of *A. labyrinthica* evidenced the importance of CAT in antioxidative reactions; the activity of this enzyme was greater in males than females. In *L. triangularis*, both CAT and GPx activity were lower than in the above epigeic species. Only in the case of GPOX did females displayed higher enzymatic activity than males. Midgut glands of *L. triangularis* also included high levels of GSH, suggesting an important contribution of this tripeptide to detoxifying reactions.[37] Although CAT and glutathione peroxidases may cooperate in cell protection against ROS, one of them is usually more active in hydrogen peroxide neutralisation, depending on the substrate concentration. High H_2O_2 concentrations activate CAT, while glutathione peroxidase is stimulated at low concentrations of the ROS, owing to the fact that glutathione peroxidase directs hydrogen peroxide towards glutathione, using NADPH; therefore, the cycle is energetically expensive. In spiders, the type of triggered defense reactions may also depend on the level of oxidative stress. Quantitative and qualitative analysis of antioxidative responses can provide a basis for comparing these predators in terms of sensitivity to chemical substances. Trends in changes in the activity of selected detoxifying, *e.g.* antioxidative, enzymes in spiders from anthropogenically changed areas compared to values recorded in individuals from reference sites are presented in Table 5.1.

Physiological and biochemical defence reactions triggered in organisms usually result in changes in cellular concentrations of adenine nucleotides (ATP, ADP, AMP). These metabolites belong to the most universal enzymatic effectors, with ATP being a positive effector for enzymes regulating anabolic pathways and AMP activating the regulatory enzymes of catabolic pathways. Concentrations of adenylate nucleotides can be used to determine the adenylate energy charge (AEC), depending on the balance between catabolic and anabolic processes. From levels of these metabolites measured in tissues of behaviourally different spider species it can be deduced that, even in conditions of strong environmental contamination with heavy metals, the cellular energy balance is not noticeably disturbed. Irrespective of species and level of pollution at study sites, AEC values in the non-web-building *P. palustris* and web-building *L. triangularis*, *A. diadematus*, and *M. segmentata* fell within physiologically optimal limits (above 0.750), although detoxifying reactions catalysed by CarE, GST, SOD, and CAT were intensified. In the most heavily contaminated region (area of a metallurgic factory), the main strategy

Table 5.1 Trends in changes in the levels of considered biochemical and cellular parameters in bodies and/or midgut glands of females (F) and males (M) of selected spider species (*X.n.*: *Xerolycosa nemoralis*; *P.l.*: *Pardosa lugubris*; *A.l.*: *Agelena labyrinthica*; *L.t.*: *Linyphia triangularis*; *M.s.*: *Metellina segmentata*; *A.d.*: *Araneus diadematus*; *P.p.*: *Pardosa palustris*) from areas affected by industrial pollution, compared to values recorded in individuals from reference sites: ↑ – increase; ↓ – decrease; ↔ no noticeable changes; [a]measurements in whole spider bodies.

Species	*X.n.*[37]		*P.l.*[48,49]		*A.l.*[37,48,49]		*L.t.*[30,37,47]		*M.s.*[30,47]	*A.d.*[30,47]	*P.p.*[30]
Sex	F	M	F	M	F	M	F	M	F	F	F
CarE			↑[a]	↑[a]	↑[a]	↑[a]	↑ ↓[a]		↓[a]	↓[a]	↑[a]
GST			↑[a]	↓[a]			↑ ↑[a]		↑[a]	↑[a]	↑[a]
SOD							↑[a]		↑[a]	↑[a]	↑[a]
CAT	↔	↑			↑	↑	↔ ↑[a]	↔	↔[a]	↑[a]	↑[a]
GPOX	↑	↑	↔[a]	↑[a]	↓ ↔[a]	↔ ↔[a]	↔	↔			
GSTPx	↔	↔	↔[a]	↑[a]	↑ ↔[a]	↑ ↔[a]	↓	↓			
GSH	↑	↑	↔[a]	↑[a]	↔ ↑[a]	↔ ↑[a]	↑	↔			
%MTs	↔	↔			↑	↑	↔	↓			
%Hsp70	↔	↑			↓	↔	↔	↑			
%Apoptosis	↑	↔			↑	↓	↔	↓			
%Necrosis	↔	↑			↓	↔	↑	↓			
%low ΔΨm	↑	↔			↓	↔	↔	↑			

used by spiders was to maintain high levels of adenine nucleotides, while at other sites affected by pollution (from coal and chemical industries) animal bodies included lower amounts of the metabolites.[30,50] In these conditions, spiders also showed enhanced activity of enzymes involved in anaerobic processes.[51] *A. diadematus* orb-web spiders and *P. palustris* wolf spiders, differing in both hunting strategy and activity, displayed elevated levels of cytoplasmic lactate dehydrogenase (LDH) and malate dehydrogenase (MDH). High MDH content was also recorded in tissues of orb-web *M. segmentata* spiders. LDH and MDH activity only decreased in the sheet-web *L. triangularis* spiders. As spiders vary in their metabolic responses to environmental pollution, their tolerance to the acting toxins seems to be species-specific, which explains the dominance of particular species in arachnocoenoses of contaminated areas.

5.2.1.3 Heat Shock Proteins and Cell Death Processes

In physiological conditions, the apoptotic mechanism is responsible for balancing the rate of cell proliferation and removal of damaged or useless cells. The process can be stimulated by various stressing factors, particularly ones increasing the risk of reactive oxygen species generation. In conditions of low ROS concentration, decreasing the ATP level temporarily, cells usually die in an apoptotic way, while high concentrations of hydrogen peroxide, decreasing the intracellular ATP resources permanently, lead to necrotic death. The intracellular ADP/ATP concentration ratio can serve as a biomarker of the cellular energetic status, allowing predictions of future events

in cells, towards proliferation or cell death processes. High values of the ratio, resulting from a decrease in the number of ATP molecules in a cell, are interpreted as deterioration of its metabolic condition. In studies of *A. labyrinthica*, a much higher ADP/ATP ratio was reported for cells of individuals exposed to cadmium and copper in laboratory conditions than for control groups. The strongest response was observed in males from polluted areas, showing ADP/ATP ratios 9-fold (Cd) or 6-fold (Cu) greater than in the control. In females treated with the same metals the changes were nearly 2-fold less intensive.[39]

Midgut glands of male *X. nemoralis*, originating from areas contaminated with zinc and lead and examined with flow cytometry, included a high percentage of necrotic cells. High concentrations of metals were also recorded in the organ. In the same conditions, female cells were affected by intensified apoptotic changes and increase in the percentage of mitochondria with low transmembrane potential ($\Delta\Psi_m$).[37] *X. nemoralis* was also typified by a high frequency of Hsp70-positive cells (Table 5.1). As induction of heat shock proteins may occur in response to natural and chemical stressing factors, it can be assumed that production of these proteins was stimulated within compensation reactions of the organism triggered owing to the strong pressure of industrial pollution. Greater Hsp70 levels in females may evidence intensified cellular defense reactions preventing degenerative changes in the midgut gland. It cannot be excluded that owing to their greater exposure to pollutants (resulting, for example, from higher hunting activity and longer life span) females take advantage of diverse defense mechanisms, even energetically costly ones, in order to maintain reproductive fitness.

Genotoxic effects of cadmium in various spider organs were also confirmed with the comet assay (single cell gel electrophoresis; SCGE). A simple food chain model: medium with metal → *Drosophila hydei* flies → *S. grossa* spiders (Figure 5.4), was used to indicate DNA damage caused by the

Figure 5.4 Adult female and male *Steatoda grossa* cobweb-weaving spider (Theridiidae).

Table 5.2 TDNA (tail DNA; %), TL (tail length; μm) and OTM (tail moment; arbitrary units) in midgut gland cells and haemocytes of *Steatoda grossa* spiders from experimental groups (C – control; Cd – exposed to cadmium);[42] F – females; M – males; Median ± quartile deviation (25th and 75th percentiles). Different letters ($^{a, b}$) indicate significant differences between experimental groups (C, Cd) within females and males; ^{c}indicates significant differences between females and males of complementary groups (Mann–Whitney U test, $p < 0.05$).

		TDNA		TL		OTM	
Material	Sex	C	Cd	C	Cd	C	Cd
Midgut gland cells	F	3.7^{a}	31.6^{b}	4.6^{a}	20.9^{bc}	0.5^{ac}	4.0^{b}
		0.7–12.9	18.7–45.0	0.0–7.1	9.8–38.5	0.1–1.2	1.8–8.7
	M	1.4^{a}	41.5^{b}	2.7^{a}	30.8^{bc}	0.1^{ac}	6.3^{b}
		0.4–6.0	20.5–55.9	0.6–4.4	21.1–41.0	0.03–0.4	2.6–9.4
Haemocytes	F	2.9^{a}	44.8^{b}	4.1^{ac}	35.4^{b}	0.3^{a}	7.9^{bc}
		0.4–10.0	30.0–62.1	0.0–7.7	21.1–49.0	0.1–0.9	3.0–13.0
	M	5.5^{a}	36.4^{b}	5.2^{ac}	27.4^{b}	0.6^{a}	5.0^{bc}
		0.9–13.1	17.3–55.5	2.7–11.9	14.0–42.0	0.1–1.7	1.8–12.1

xenobiotic both in midgut gland cells and haemocytes. DNA damage in the analysed cells most likely resulted from the activity of assimilated cadmium that was not deposited in granules, not bound to, *e.g.*, metallothioneins or even mobilized in metabolic processes. Genotoxic damage was larger in haemocytes than midgut gland cells. Therefore, it can be assumed that, even at low concentrations, cadmium may negatively affect haemolymph cells. The level of DNA damage in *S. grossa* spiders was also sex-dependent. In midgut gland cells of males, the recorded genotoxic effects were stronger than in similarly treated females. It cannot be excluded that females have more effective cadmium-neutralizing mechanisms limiting the metal toxic action in midgut gland cells[42] (Table 5.2).

5.3 Spider Sensitivity to Pesticides

In agrocoenoses, where spiders are considered to support humans in crop pest control, reduction in population sizes of these predators may result in expansion of herbivores.[2,5] The efficiency of spiders in decreasing the numbers of plant pests was often comparable with the effects of plant protection agents.[52,53] Therefore, high numbers of these predators maintained in agricultural ecosystems may provide a safe alternative to synthetic insecticides.[54]

Though spiders are usually not a direct target of plant protection agents, their incidental, but still negative, effects are often observed in the predators. The harmful impact of pesticides results from both their immediate toxicity, causing death or metabolic and physiological disturbances,[55–59] and the drastic decrease in prey availability and consequent prolonged starvation periods.[2,60,61] It has been experimentally confirmed that plant protection

agents, the remains of which show long-lasting toxicity in environments, may cause long-term disturbances in spider behaviour and therefore hinder restoration of arachnocoenose size in agroecosystems.[62] Some studies have also shown that parental generations exposed to pesticides produce offspring more sensitive to potential future contact with such compounds. In a longer perspective, this may decrease the efficiency of spiders as natural regulators of pest population sizes in agricultural areas.[63]

It has been documented that spiders are able to gradually recolonise polluted agrocoenoses, though being affected by plant protection agents.[53,64,65] Numerous field and laboratory tests indicate that the predators vary in their sensitivity to pesticides applied in agrotechnical measures, depending on the type of chemical compound, sex of the individuals, feeding strategy and season.[66–70] Generally, spiders tolerate fungicides and herbicides better than insecticides.[71,72] Nevertheless, even in the case of the last-mentioned ones, spider tolerance is species-specific. For example, *Hibana velox* (Anyphaenidae) spiders showed 100% mortality after exposure to even low doses of chlorpyrifos and ethion (organophosphorus insecticides), carbaryl (carbamate insecticide) and dicofol (organochlorine pesticide), while they were not affected by the toxicity of other compounds, such as azadirachtin (neem) and diflubenzuron (benzoylurea-type insecticide), even if applied at high concentrations.[68] Alphamethrin (pyretroid) and endosulfan (organochlorine insecticide) also appeared to be tolerated differently by four spider families, Lycosidae, Clubionidae, Theridiidae and Linyphiidae, dominant in South African cotton fields. Among web-building spiders, Linyphiidae were less resistant to the pesticides than Theridiidae. When considering non-web-building species, the compounds did not affect the size of Lycosidae populations. The number of Clubionidae was strongly reduced owing to exposure to both pesticides, and only in endosulfan-treated areas the populations could be quickly restored. It cannot be excluded that the lower sensitivity of particular spider groups resulted from their behaviour and avoidance of direct contact with pesticides.[66] Importance of the foraging mode in development of tolerance to plant protection agents was also confirmed in analyses of toxic effects of three commercial insecticides, an insect growth regulator (hexaflumuron), a selective organophosphorus (phosalone) and a non-selective pyretroid insecticide (permethrin), in behaviourally different species, the web-building *Araniella opisthographa, Dictyna uncinata* and *Theridion impressum*, as well as spiders not spinning hunting webs, *Pardosa agrestis* and *Philodromus cespitum*, also representing various lifestyles (nocturnal/diurnal). The actively hunting spiders appeared to be more sensitive to the applied compounds than species building hunting webs. Moreover, it was observed that diurnal hunting spiders (*Philodromus* and *Pardosa*) were severely affected only by permethrin, while nocturnal hunting spiders, *Clubiona neglecta*, displayed high mortality after exposure to phosalone and permethrin.[73]

Sensitivity to the considered pesticides was also sex-dependent. Males of *Rabidosa rabida* wolf spiders (Lycosidae) treated with sublethal doses of the organophosphorus malathion showed disrupted mating behaviour, which

resulted in most dosed males being killed by females without achieving copulation.[70] In *P. amentata* wolf spiders, irrespective of sex, application of cypermetrin caused symptoms of ataxia and hind legs paralysis, observed for maximally 3–6 days. However, females of *P. amentata* exposed to the compound and starved lived longer than males.[69] In the same species, use of λ-cyhalotrin (acaricide) resulted in nearly 50% mortality of males, while the percentage of dying females was over 2-fold lower.[67] It has been also reported that *P. amentata* affected by the acaricide during late autumn or early winter were relatively less sensitive to its action than in spring and summer, when, at the same level of exposure, individuals showed high mortality. Increasing sensitivity of lycosid spiders to the pesticide most likely corresponds to their intensified activity, providing more frequent contact with the chemical stressor, in search for sexual partners during the reproductive period. The physiological condition of predators may also have been worse, as males most often die shortly after copulation. Females, owing to the need to maintain intensive detoxifying reactions, may have decreased their energy expenditure for cocoon production, resulting in lower numbers of hatching young spiders.

5.3.1 Changes in AChE Activity

Most plant protection agents, though showing different chemical structures and aimed at various targets, act neurotoxically. In spider organisms, such effects can be manifested as locomotor dysfunctions, namely, depending on the dose of pesticide, reduction or induction of movement, eventually disturbing the hunting behaviour of individuals.[69,74] In the case of organophosphorus pesticides, motor disturbances in spiders usually result from synaptic transmission changes based on inhibition of acetylcholinesterase (AChE) activity.[40,63,75,76] Comparison of dimethoate's (organophosphorus insecticide) influence on *A. labyrinthica* funnel-web spiders and *P. lugubris* wolf spiders shows that both single and multiple applications of the compound in sublethal doses suppressed AChE activity in agelenid spiders; however, in *P. lugubris* it caused only weak effects.[76] These results are similar to those obtained for *P. lugubris* after exposure to another organophosphorus pesticide, fenitrotion, which did not cause noticeable changes in AChE levels in either females or males.[40] In *Lycosa hilaris* wolf spiders (Lycosidae), treated with diazinone and chlorpyrifos in laboratory conditions, cholinesterase (ChE) activity was inhibited to respectively 14% and 61% of control values. The compounds showed greater toxicity in males than females. In mesocosm investigations of the species, the surviving diazinone-exposed individuals displayed 87% suppression of ChE activity, however returning to the control level 8 days after pesticide application.[75] In studies of adult linyphiid spiders *Hylyphantes graminicola*, subjected to separate and combined action of fenvalerate (pyretroid) and dimethoate, AChE inhibition was confirmed, however in combination the pesticides produced a synergic effect.[63]

5.3.2 Enzymatic Detoxifying Reactions

In spiders, contact with plant protection agents triggers detoxifying reactions of both the first and second phase of xenobiotic biotransformation processes. For example, consecutive development stages of *Pardosa pseudoannulata* (Lycosidae) responded to the trichlorfon pesticide with increased concentrations of esterase isoenzymes.[77] Treatment with cypermetrin (pyretroid), introduced *via* contact, in two species differing in their hunting strategies, *P. prativaga* wolf spiders (Lycosidae) and *L. triangularis* sheet-web spiders, intensified the detoxifying reactions associated with glutathione metabolism. Nevertheless, the species varied strongly in their ability to detoxify the pesticide.[78] *L. triangularis* showed high basal GST activity, tending to be induced during further exposure to the compound, but low activity of GPx, participating in antioxidative reactions. *P. prativaga* ground-dwelling spiders displayed low GST activity and GPx activity significantly increasing after 12 hours from exposure to the pesticide. No noticeable changes in GST activity were observed in *L. hilaris* wolf spiders after application of diazinone and chlorpyrifos, and in *P. amentata* treated with cypermetrin.[75,79] Additionally, the last-mentioned species responded to the pesticide with fluctuating GPx activity, depending on the studied season, being high during winter and low (though susceptible to induction) during full activity of animals.[79] Sublethal doses of dimethoate also enhanced SOD and CAT activity in the midgut gland of *P. lugubris.* In contrast, in *A. labyrinthica* affected by a chemical stressor, levels of these enzymes did not significantly change or even, in case of CAT, decreased in comparison with the control.[80]

Analysis of changes in the activity of antioxidative enzymes in spiders also revealed sex-specific differences in the ability to tolerate low pesticide doses. In *X. nemoralis* (Figure 5.5), five-time application of sublethal dimethoate doses, administered topically every 24 h, enhanced CAT activity in male midgut glands as well as increasing the concentration of GSH and the

Figure 5.5 The non-web-building *Xerolycosa nemoralis* wolf spider (Lycosidae).

activity of enzymes associated with the metabolism of this tripeptide (GPOX, GSTPx, GST). In the same conditions, females showed high SOD and CAT activities in the organ. Similar effects were noted for high-temperature stress and combined action of heat shock and dimethoate.[81] It cannot be excluded that in females, who need to save energy as, in comparison with males, they have a longer life span and expend greater reproductive energy costs, CAT enabled a more effective antioxidative defence.

Inter-sex differences in sensitivity to dimethoate were confirmed in analyses of antioxidative response in spider haemolymph. Levels of GSH, GSTPx, CAT and total antioxidant capacity (TAC) measured in *X. nemoralis*, subjected to contact exposure to the organophosphorus insecticide, indicated that the compound caused stronger oxidative stress in males than females.[82] Irrespective of dose, in females the activity of CAT, GSTPx and glutathione reductase (GR) did not change as compared to the control. A different pattern was observed in males, in which CAT activity increased after a single application of a sublethal dimethoate dose and significantly decreased, attaining control values, after five times of application. These changes were accompanied by high GR activity, likely to result from greater demands for reduced glutathione in male organisms. The tripeptide not only protects the functions of enzymes, *e.g.* GPx and GST, but may be also involved in antioxidative reactions, such as reduction of endogenous hydrogen peroxide and neutralization of superoxide anion radicals and hydroxyl radicals, or directly bind to xenobiotics and their metabolites. Trends in changes in the levels of considered biochemical and cellular parameters in dimethoate-treated spider females and males are presented in Table 5.3.

5.3.3 Genotoxic and Cytotoxic Effects of Plant Protection Agents in Spiders

The available literature does not provide much data on the genotoxic effects of pesticide action in spiders. The comet assay used in investigations of DNA damage induced by chlorpyrifos (organophosphate) and acetamiprid (neonicotinoid), administered in various concentrations, confirmed the genotoxic effects of applied insecticides in *Pardosa astrigera* (Lycosidae). The study revealed significant differences in the proportion of cells with DNA tails and the tail length of nuclear DNA between different concentrations of the considered pesticides.[83] In *X. nemoralis*, the level of DNA damage in midgut gland cells and haemocytes of females and males, subjected to single or five-time (simulation of chronic exposure) application of sublethal dimethoate doses, appeared to be sex-dependent. In response to the used organophosphorus compound, the two cell types displayed stronger genotoxic effects in males than in females. After five-time exposure to the pesticide, level of DNA damage exceeded control values in cells of males, but in females the genotoxic effects were weak. Females of the species are likely to take advantage of cellular repair mechanisms more effectively in protecting

Table 5.3 Trends in changes in the levels of considered biochemical and cellular parameters, in response to chronic contact with dimethoate, in females (F) and males (M) of spiders from areas affected by industrial pollution (P) and reference sites (UP): ↑ – increase; ↓ – decrease; ↔ no noticeable changes; [a]measurements in whole spider bodies.

Species	*X. nemoralis*[81,82]						*P. lugubris*[76,80]		*A. labyrinthica*[76,80]	
Material	Midgut glands				Haemolymph		Midgut glands		Midgut glands	
Sex	F		M		F	M	F		F	
Site	P	UP	P	UP			P	UP	P	UP
SOD	↑	↔	↔	↑			↑	↔	↔	↔
CAT	↑	↔	↑	↑	↔	↔	↑	↔	↓	↔
GPOx	↓	↔	↑	↑			↑[a]	↔[a]	↓[a]	↓[a]
GSTPx	↓	↔	↑	↑	↔	↔	↔[a]	↔[a]	↓[a]	↓[a]
GSH	↔	↔	↑	↑						
GR					↔	↑				
GST	↔	↑	↑	↔			↔[a]	↔[a]	↓[a]	↔[a]
TAC					↔	↑				
CarE							↓[a]	↔[a]	↓[a]	↓[a]
AChE							↔[a]	↔[a]	↓[a]	↓[a]
%MTs	↑	↑	↔	↑			↑	↑	↑	↑
%Hsp70	↔	↔	↑	↑			↑	↑	↑	↑
%Apoptosis	↔	↑	↑	↑			↔	↔	↑	↑
%Necrosis	↑	↑	↔	↑						
% low ΔΨm	↑	↑	↑	↑			↔	↔	↑	↑

Table 5.4 TDNA (Tail DNA; %), TL (Tail length; μm) and OTM (Tail moment; arbitrary unit) in midgut gland cells and haemocytes of *Xerolycosa nemoralis* (Lycosidae) spiders from experimental groups (C: control; Dimethoate: exposed to the pesticide);[84] F: females; M: males; Median ± quartile deviation (25th and 75th percentiles). Different letters ($^{a, b}$) indicate significant differences between experimental groups (C, Dimethoate) within females and males; ^{c}indicates significant differences between females and males of complementary groups (Mann–Whitney U test, $p < 0.05$).

		TDNA		TL		OTM	
Material	Sex	C	Dimethoate	C	Dimethoate	C	Dimethoate
Midgut gland cells	F	5.5^{a}	5.2^{ac}	4.7^{a}	7.9^{bc}	0.6^{a}	0.5^{ac}
		0.6–20.7	0.7–19.7	0.0–9.8	3.5–46.2	0.1–1.8	0.1–1.5
	M	7.4^{a}	12.1^{bc}	6.9^{a}	30.7^{bc}	0.6^{a}	1.2^{bc}
		0.9–22.7	0.7–53.8	2.7–19.9	6.7–48.3	0.1–2.4	0.1–9.4
Haemocytes	F	25.0^{ac}	14.1^{ac}	24.2^{ac}	32.3^{ac}	3.1^{ac}	1.7^{ac}
		9.5–54.2	3.6–39.0	7.1–38.9	5.8–55.2	1.5–10.2	0.4–7.1
	M	6.3^{ac}	35.0^{bc}	9.2^{ac}	44.3^{bc}	0.6^{ac}	6.1^{bc}
		1.3–20.7	12.5–74.5	1.5–24.5	30.5–64.0	0.1–2.8	1.8–14.3

the genetic material from damage and therefore have a greater capability to survive in conditions of incidental exposure to such compounds than males. Irrespective of sex, haemocytes of *X. nemoralis* were more sensitive to DNA damage than midgut gland cells. Consequently, these haemolymph cells can the used in biomonitoring studies to assess the toxic effects of low concentrations of chemical substances on these invertebrates (Table 5.4).[84]

Cellular effects of dimethoate in spiders were also reflected by stimulation of cell death processes. *A. labyrinthica* spiders exposed to the insecticide showed greater numbers of cells with depolarised mitochondria (low $\Delta\Psi_m$) and more frequent apoptotic changes in midgut glands. However, in *P. lugubris* both parameters were recorded in low values, what confirms the species-specific sensitivity of the predators to the pesticide.[80]

In midgut glands of female *P. lugubris*, low frequency of apoptotic changes was accompanied by intensive production of proteins, MTs and Hsp70, and high activity of SOD and CAT. In dimethoate-treated *A. labyrinthica*, a nearly 10-fold rise in the number of Hsp70-positive cells was also observed, but SOD and CAT values remained at a constant level. The above-mentioned components of the antioxidative system in *P. lugubris* wandering spiders were likely to effectively protect their cells from degenerative changes and contribute to the development of tolerance to environmental factors. Application of dimethoate also caused degenerative changes in midgut gland cells of another Lycosidae species, *X. nemoralis.*[85] However, the intensity of apoptotic and necrotic changes in individuals depended on their earlier preexposure to industrial pollutants and was high in spiders from the reference site. Irrespectively of sex and life history of *X. nemoralis* individuals, after exposure to dimethoate they showed a significant increase in the percentage of mitochondria with low $\Delta\Psi_m$. The obtained results confirm

the usefulness of this parameter as a biomarker of early, subcellular effects of pesticides in spider cells.

5.4 Starvation Stress

As predatory invertebrates, spiders are often exposed to periodic food deficiency in their habitats, so they show high tolerance to this stressing factor. Previous studies of this issue were focused on behavioural and physiological adaptations to survival of starvation periods rather than on their consequences at the cellular level. It has been documented that spider tolerance to starvation depends on the season as well as the development stage and sex of the individual.[86–88] One way in which spiders cope with food deprivation is the sit-and-wait strategy, in which the problem is somehow solved by spending the starvation period motionless, until the prey arrives. Such a tactic, in which energy expenditure can be reduced, is an alternative to searching for a new habitat.[88] In prolonged starvation periods, spiders cease their activity, use the stored energy reserves to maintain basal metabolism and do not increase their body size in order to survive. However, even in such adverse conditions they can still reproduce, though the number and mass of eggs laid is low; small and underfed females are less fertile and delay the cocoon production period.[88,89]

Spiders are particularly prone to prolonged starvation periods in contaminated areas as chemical compounds introduced to the environment can drastically decrease prey availability. In such conditions, survival may depend on the nutritional condition and amount of energy reserves deposited by the individual. *P. prativaga* wolf spiders that obtained nutrient-rich food were more resistant to both starvation and contact with dimethoate than individuals kept on a low-quality diet or starved.[90] In the species, life span of spiders deprived from food depended on their fat reserves and was the longest in individuals provided with lipid-rich food in the pre-starvation period.[91]

Spiders show strong sexual dimorphism, manifested in body build and composition as well as physiological and behavioural characteristics. Generally, females are larger, have a longer life span and expend greater reproductive effort in comparison to males. Moreover, females usually accumulate more lipids than males, which has been indirectly indicated in analyses of the dry mass/wet mass ratio, *e.g.* in *Hygrolycosa rubrofasciata* wolf spiders, or water content in *Lycosa ceratiola*.[92,93] The sexes also appear to significantly differ in their energy consumption. Females of *P. lugubris* wolf spiders show a nearly 2-fold increase in body mass in the period preceding cocoon production, while males retain a low and relatively stable body mass until death.[94] In conditions of food deprivation, females can also reduce their metabolic rates to a greater extent than males and therefore survive much longer starvation periods.[87,89,93]

Starvation induces strong morphological changes in the midgut gland of spiders. In starved *Coelotes terrestris* funnel-web spiders (Agelenidae),

digestive cells of the organ displayed decreased levels of glycogen and lipids accompanied by increased numbers of secretory vacuoles, expanded in the greatest part of the cell. It cannot be excluded that such changes resulted from autophagy, activated *inter alia* in response to nutrient deficiency in cells.[25]

Stress induced by nutrient deficiency or complete food deprivation, being a strong prooxidative factor, is likely to determine cellular defense responses to anthropogenic stressing factors. *P. prativaga* spiders fed with low-quality prey for 2–4 weeks, compared to individuals kept on a high-quality diet, showed suppressed activity of glutathione peroxidase and unchanged GST activity.[95] Other studies documented more frequent degenerative changes in midgut glands of starved *X. nemoralis,* suggesting that this type of stress strongly affects the metabolic processes of the considered organ. Irrespective of sex, the species displayed enhanced apoptotic, and in case of males also necrotic, processes.[85] In starvation periods, midgut glands of *X. nemoralis* females showed elevated concentrations of glutathione and MTs as well as intensified SOD, CAT, GPOX, and GSTPx activity. In males, starvation resulted only in increased CAT and glutathione peroxidase activity; levels of other antioxidative parameters, including heat shock proteins, remained unchanged or became even lower.[81] These results indicate that males, with their small energy reserves, which are even more limited in the reproductive period owing to ceased hunting activity, are particularly prone to the effects of oxidative stress if exposed to additional stressors. However, stimulation of both enzymatic and non-enzymatic antioxidants in response to starvation stress effectively protects the females from oxidative stress and degenerative changes in organisms.

Starvation stress did not cause genotoxic changes in female *X. nemoralis*. High level of DNA damage recorded in both haemocytes and midgut gland cells of males indicates that prolonged starvation periods may be a strong stressor for the individuals, decreasing their capability for survival, particularly if additional stressors are encountered. In males, prolonged starvation periods may strongly suppresses immunity and make individuals more prone to microbial invasions.[82,84]

5.5 Conclusions

In spiders, cellular response to stress induced by direct and indirect anthropogenic factors is species- and sex-specific. Therefore, results obtained for one species should be extrapolated to other ones only with a dose of reserve. Different detoxifying reactions observed in female and male spiders indicate that the sexes use different compensation strategies to survive long-lasting stress. Therefore, in studies of these predatory invertebrates the effect of gender must be considered. Analyses carried out on both sexes together may not reveal the relationship between the intensifying stressing factor and level of enzymatic and non-enzymatic cellular response.

Biochemical and cellular parameters examined in spiders can be used as nonspecific biomarkers, allowing detection of adverse processes in the organism, but not precise identification of their underlying mechanisms. Quantitative analyses of changes in the levels of cellular indicators enable comparisons of sensitivity to chemical factors between spider species. Moreover, such investigations may allow identification of defense strategies triggered in the predators by different types of environmental stressors and prediction of the species' ability to occur and survive in polluted areas.

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Section II: Terrestrial Vertebrates as Experimental Models

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CHAPTER 6

Use of Melanin-pigmented Cells as a New Tool to Evaluate Effects of Agrochemicals and Other Emerging Contaminants in Brazilian Anurans

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6.1 Color in Animals

Specialized cells for storing pigments, chromatophores, are found in invertebrate and vertebrate ectotherms. These cells show various cytoplasmic projections and are originated from the embryonic neural tube; then, they migrate to the skin and are distributed on the epidermis and dermis.[1]

There are various types of pigments found in chromatophores. In vertebrates, at least five of them have been described. Melanophores, which are black or brown, contain melanin in granules. Erythrophores, with their reddish color, contain pteridine; xanthophores also have pteridine, besides carotenoids located in vesicles, which give them a yellow color. Iridophores

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have a metallic color, owing to the presence of purines deposited in reflecting crystals. These four types of pigmented cells are found in fish, amphibians, and reptiles. Leucophores, on the other hand, contain purine granules, which give them a white color, and are found only in fish.[1,2] Our main analyses and observations are based on a very common type of extracutaneous melanophore, called visceral melanocyte, which is discussed further on (Figure 6.1).

Even though the term melanophore is widely used by several authors,[3] in order to simplify the nomenclature and to recognize the increasing evidence of a conservation genetics of the melanocytes biology, the term melanocytes

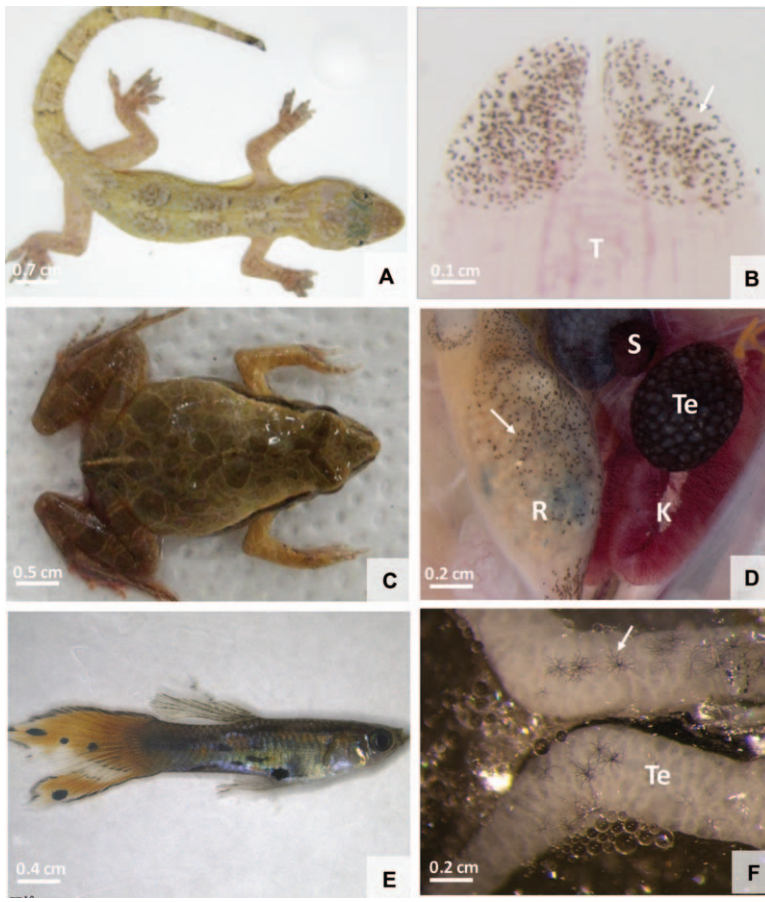

Figure 6.1 External and visceral colors in ectothermic animals (A, B: Reptile, *Hemidactylus mabouia;* C, D: Anuro, *Physalaemus cuvieri,* E, F: Fish, *Poecilia reticulata* and *Knodus moenkhausii*). Different color patterns given by chromatophores (A, C, E) besides the presence of visceral melanocytes (arrows) with a dendritic aspect, which give the organs a dark color. A: *H. mabouia* natural color. B: Melanocytes present (arrow) in the ventral surface of the tongue tip. C: *P. cuvieri* color, showing the coloration pattern. D: Cells containing melanin (arrows) in organs. E: Cutaneous color of *P. reticulata.* F: Presence of pigmented cells in *K. moenkhausii* testes. K: Kidney. R: Rectum. S: Spleen. T: Tongue. Te: Testes.

was proposed to be applied to all those cells. However, since the term melanophore has been used by different authors and it is also correct, it may still be used.

An advantage in introducing this additional category of melanocytes (melanophores) is to create a more precise classification of the cells' behavior, but it has not been incorporated yet, such as by other subtypes of melanin-secreting melanocytes in mammals, which have not been treated similarly. We opted to use the term melanocyte for cells that contain melanin in the internal organs of ectothermic animals and melanophore for melanin-containing cells in skin.

Cutaneous chromatophores are usually found on the dermis, where erythrophores are the most superficial and melanophores are the deepest ones (Figure 6.2). The arrangement of these pigment cells over the different

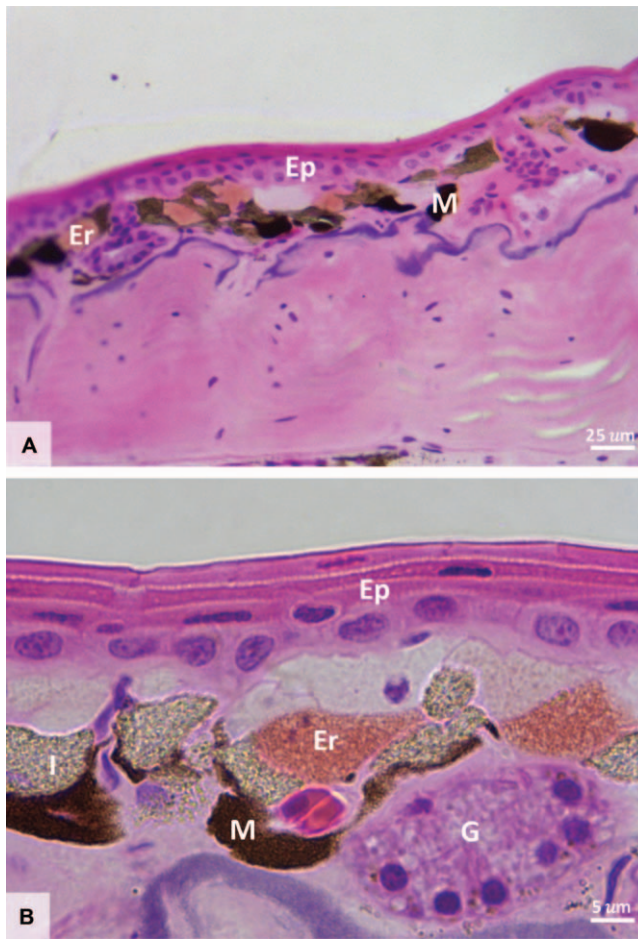

Figure 6.2 Histology of *Physalaemus nattereri* skin. A: General view of the skin section showing an erythrophores layer (Er), found under the epidermis (Ep), followed by a deeper melanophores layer (M). B: Chromatophores found in the skin: erythrophores (Er), iridophores (I) and melanophores (M), with their different colors. G: exocrine gland.

skin layers is related to the type of pigment they contain and which wavelengths they reflect and absorb, thus providing vertebrates with different colors.[2]

In fish, some chromatophores respond to changes in the environment, which might interfere with the animals' capacity to interact with their environment (*e.g.*, camouflage, cryptic colorations). Recently, authors[4] have shown that in fish (*Oreochromis mossambicus*) melanophores respond to lead nitrate, phenol, and hexachlorocyclohexane. In another fish, *Channa punctatus*, upon exposure to arsenic, the dispersion of melanophores was decreased at initial exposure times (*i.e.*, up to 60 days). As a result, the animal's color becomes lighter; however, after 90 days, dispersion increases, and the animal's color becomes darker. This shows that the fish develop physiological response mechanisms to the exposure to arsenic.[5]

According to this approach, in ectothermic animals, the chromatophores that synthesize dark pigments, called melanocytes, are not only limited to the dermis, but frequently occur in connective tissue and in internal organs, such as the liver, kidneys, heart, thymus, gonad, besides blood vessels, peritoneum and meninges.[2,6]

6.2 Internal Melanin-pigmented Cells

Both fish and amphibians have pigmented cells in their organs and membranes, called internal melanocytes.[7–10] These cells are originated from the ectothermic neural crest;[11] they contain large quantities of melanin[6] and are similar to dermal melanocytes.[12] They also have various dendritic extensions, which can even be observed without a microscope.[13]

There are other, different, pigmented cells called melanomacrophages, which are present in hematopoietic organs (*e.g.*, liver and spleen) and have phagocytic activity similar to macrophages.[14,15] These cells have substances from cellular catabolism in the cytoplasm, such as hemosiderin and lipofuscin.[14] Melanomacrophages are derived from hematopoietic stem cells and are round in shape.[11,16]

In common, and our main interest in this chapter, is the melanin present both in melanocytes and melanomacrophages; it is a complex polymer synthesized endogenously in vertebrates and invertebrates[17] that absorbs and neutralizes free radicals and other potentially toxic agents resulting from catabolism.[14] Furthermore, melanin plays an immune role in ectothermic animals, owing to the action of hydrogen peroxidases and their quinone precursors, which act as bactericides through the increase in enzymatic activity, even though this is restricted to low temperatures.[18] Another function attributed to melanin is related to photoreception and thermoregulation in ectothermic animals, besides photoprotection, acting as a photosensibilizer in cells exposed to radiation with enough energy to cause genetic damage.[19]

In this context, for example, for certain anuran amphibians, both testicular melanocytes and hepatic melanomacrophages respond to the administration of lipopolysaccharide (LPS) from *Escherichia coli* with an

increase in the pigmented area, which suggests that these cells have tissue protection and bactericide functions.[13] The testicular pigmentation occurs in some species and, from the evolutionary and phylogenetic relationship points of view, it also varies among anuran families.[20] This internal pigmentation is a characteristic conserved throughout phylogeny in all organs.[21]

Visceral pigmentation in fish, in turn, from a functional point of view, might be related to the accumulation of residual melanin; it could also play a role in the innate immune system, have antioxidant functions, and protect the tissue against damages in the DNA.[20] However, visceral pigmentation functions in fish and anurans have been little studied and it is necessary to deepen this investigation in order to test how such pigmentation responds to external stimuli, since both transparent and non-transparent animals show internal organ pigmentation.[20]

6.3 Environmental Contamination and Its Effects on Visceral Pigmentation

Fish and amphibians live in aquatic environments, where contaminants are frequently found. Thus, these animals represent good indicators of environmental quality.[22,23] Some characteristics, such as skin permeability, shell-less eggs, exposed embryogenesis, free aquatic larvae and dependency on water for reproduction, expose amphibians to contaminants.[22,24] Fish are also excellent indicators of environmental integrity owing to their constant exposure to the aquatic environment and their different responses to environmental variations, such as habitat changes, presence of contaminants, and temperature variation.[25]

These animals' dependency on aquatic environments makes them susceptible to different contaminants, for example, pesticides and pharmaceutical drugs.[22,26,27] Contaminants present in the water may contribute in different ways to the decline of amphibians, to the impairment of the immune system, thus causing hermaphroditism, delaying both larvae growth and development, promoting morphological, physiological and behavioral changes, besides decreasing reproductive fitness or even causing sub-lethal or lethal effects.[28–30]

6.4 Response of Cutaneous Melanocytes to Aquatic Contaminants

Some descriptive studies from the 1980s showed effects of pesticides on the morphology and physiology of tadpoles' skin melanocytes. According to these studies, there is an increase in cytoplasmic extensions and thus animal color.[31,32]

However, for anurans native to neotropical regions, particularly Brazil, there are no accounts of the effects of environmental contaminants in adult skin. For larvae, an acute exposure study with *Rhinella schineideri* tadpoles in

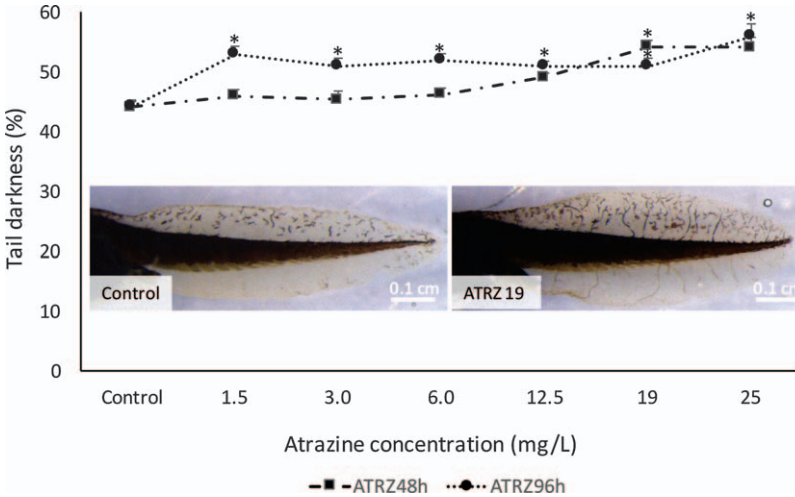

Figure 6.3 Percentage of darkening in *Rhinella schneideri* tadpoles' tails after being exposed to different concentrations of atrazine for 48 and 96 hours. In animals exposed to atrazine, after 96 hours the dispersion of melanin granules happens in low concentrations (1.5 mg L^{-1}).

contact with atrazine herbicide (concentrations 1.5 to 25 mg L^{-1}) showed that the tadpoles' bodies darkened depending on both the concentration and exposure time (Figure 6.3). In lower concentrations, darkening is only seen after 96 hours (*e.g.*, 1.5 mg L^{-1}); in higher concentrations, (*e.g.*, 12.5 mg L^{-1}), darkening is observed after 48 hours' exposure. These dispersion responses of cutaneous melanocytes regarding the herbicide exposure were similar to the response observed in anurans exposed to UV radiation,[33] thus showing that pigmented cutaneous cells are able to respond to environmental changes and may be used as morphological biomarkers for effects.

6.5 Response of Internal Melanocytes to Aquatic Contaminants

Anurans' internal melanocytes are responsive to environmental changes, such as temperature variation, UV incidence, presence of endocrine disruptors (*e.g.*, steroid hormones). These cells have some functions related to the protection of the tissue where they are found; these functions are attributed to melanin's properties.

Melanocytes found in testes, owing to the presence of a large amount of melanin, provide the gonads with a dark color in some species (Figure 6.4); others have much smaller amounts, whereas for most anuran species, testes are deprived of these pigmented cells. These pigmented cells are found on the tunic that covers the organ as well as around the seminiferous locules; melanocytes are found in association with blood vessels and show

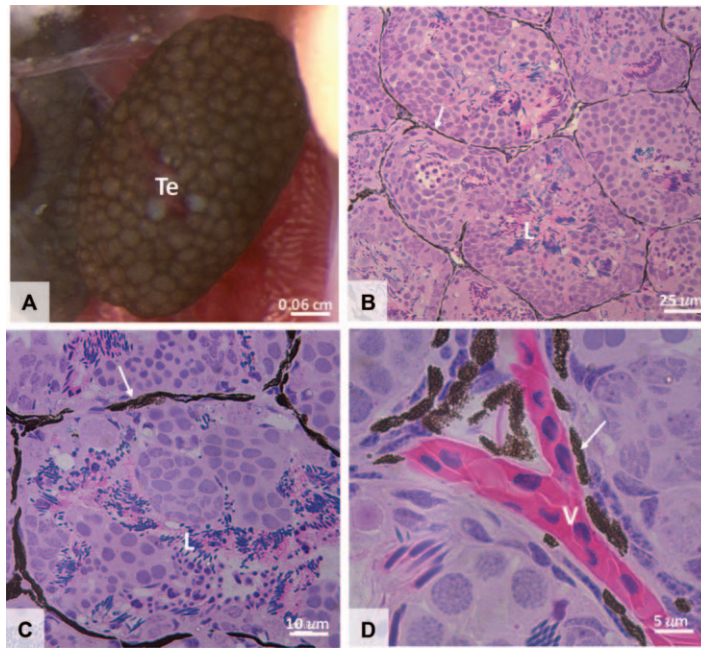

Figure 6.4 Melanocytes in *P. cuvieri* testes (Te), giving the organ a dark color (A). Melanocytes (arrows) are found around the seminiferous locules (L), as observed in B and C, D and are associated with blood vessels (V).

cytoplasmatic extensions (Figure 6.4). In order to quantify the dispersion of melanin in pigmented cells in tissues, the area occupied by melanin was used and it was measured with Image Pro Plus 6.0 software, applying the color differentiation tool.[34] Through this method, it was possible to infer the difference between treatments.

In anurans found in anthropic areas with agricultural waste, it is possible to observe an increase in the melanin-occupied area in testes, if compared to the same species in preserved environments where human impact is minimum. When comparing the melanin-occupied areas in testes of *Physalaemus cuvieri* from two different regions, one in an agricultural matrix (Rio Verde, Goiás) and another from a preserved region (Parque Nacional das Emas, Goiás), a 50% higher rate of melanin-occupied area was observed in the testes of animals in the preserved region compared to those from the agricultural matrix (Figure 6.5). In this same organ, environmental contamination can also decrease the sperm rate in the testes. The interference of environmental contamination with anurans' testes has already been described; it causes female germ cells to appear in testes of *Physalaemus nattereri*.[35] Experiments on *Bufotes variabilis* exposed to carbaryl for 96 hours showed negative effects on male germ cells.[36] However, there is not much information on the effects of aquatic contaminants in testicular melanocytes; it is known that the LPS from *E. coli* causes an acute increase in the melanocyte-occupied area after

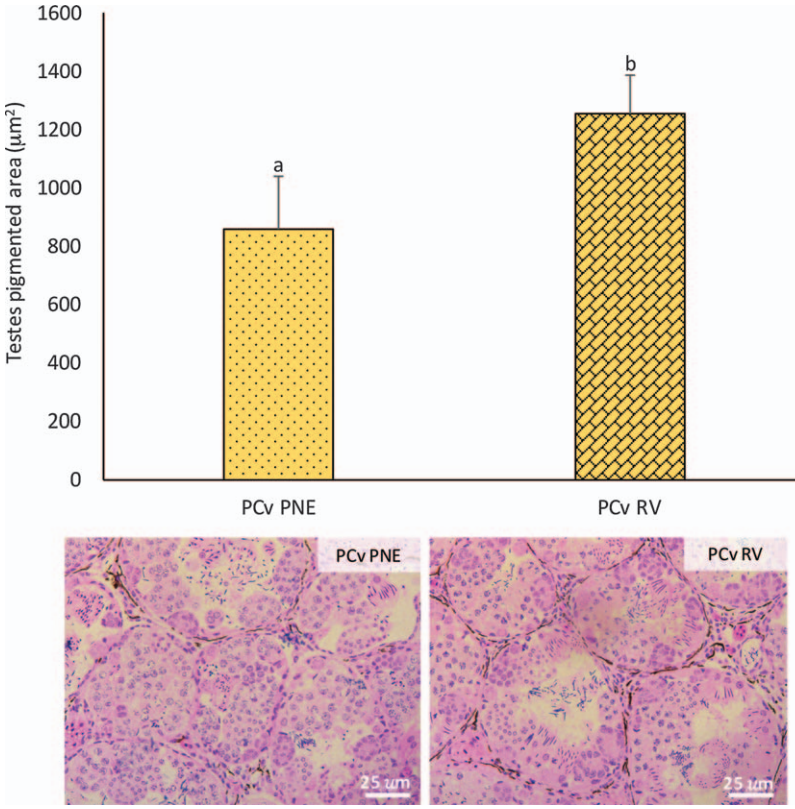

Figure 6.5 Testicular pigmentation in *P. cuvieri* collected from a preserved (PCv PNE) and from an agricultural area (PCv RV). Animals found in an agricultural matrix have a bigger testicular pigmentation area. Different letters represent the differences among the sampling environments.

2 hours of exposure, besides a change in germ cells.[13,37] Other studies have assessed the interaction between testicular melanocytes and steroid hormones (estradiol and testosterone) with an increase in occupied area after an 8 day treatment with testosterone and after an 8 day treatment followed by a 15 day recovery for animals treated with estradiol, thus showing that hormones may continue to cause effects, even a long time after exposure.[38] The presence of synthetic hormones and compounds that act as endocrine disruptors causes effects that may be systemically observed in anurans, including in their internal pigmented cells.

Nevertheless, when anurans (*Physalaemus cuvieri* and *P. nattereri*) are exposed to other classes of chemical compounds (*e.g.*, PAHs), among them benzo[*a*]pyrene (BaP) and under laboratory conditions, there is a decrease in the melanin-occupied area in testicular melanocytes in both species. In *P. nattereri*, we observed a decrease of approximately 20% in the melanin area after 3 days of exposure to BaP. In *P. cuvieri*, the reduction was

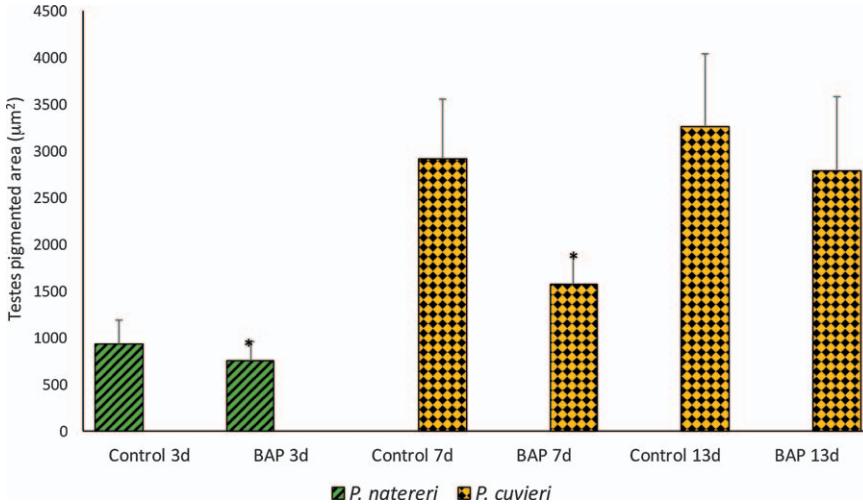

Figure 6.6 Testicular pigmentation in *P. nattereri* exposed to 3 mg kg^{-1} benzo[alfa]-pirene (BaP); and *P. cuvieri* exposed to 2 mg kg^{-1} BaP. There was a decrease in area with melanin in animals exposed to BaP after 3 and 7 days. (*) represents the difference among treatments at the same experimental time. Control 3d, 7d and 13d: Animals administered mineral oil for 3, 7 and 13 days respectively. BAP 3d, 7d and 13d: Animals administered BAP for 3, 7 and 13 days, respectively.

approximately 46%, and it happened during the 7 days of treatment (Figure 6.6). After exposure to BaP for 13 days, no effects of the treatment were observed. The BaP effect on testicular melanocytes is similar to the one observed in cutaneous melanocytes in *Xenopus laevis* cultures when exposed to glyphosate formulations. There is an inhibition of the intracellular transport because of changes in their cytoskeleton, possibly interfering in the balance of cytoplasmic calcium ions.[39] Other hypotheses referring to changes in melanin production pathways have not been tested yet, but could be influenced by BaP contamination as well.

The different responses from testicular melanocytes observed in the same species (*i.e.*, *P. cuvieri*) to different types of conditions involving environmental contaminants may be related to the cell's intrinsic properties themselves and to the mechanism they have regarding their inductors. Yet, it is believed that after such exposure a cascade of systemic effects may occur, culminating in changes in the animal's physiology, consequently affecting the organ and resident pigmented cells, metabolically disabling them, in order to fight the injury-causing agents. On the other hand, testicular melanocytes' responsiveness has been found both in animals directly originating from natural environments, where a wide array of interfering chemical substances are found, and in those under laboratory conditions, where experiments with the application of a single compound proved cause and effect.

6.6 Response of Melanomacrophages to Aquatic Contaminants

Melanomacrophages (MMs), as previously described in this chapter, are cells containing melanin; they are present in hematopoietic organs of fish and anurans. These cells, similarly to melanocytes, are sensitive and are activated with environmental changes, such as temperature variation[34,40] and UV radiation,[33] and they also act as environmental contamination indicators.[22,39]

Hepatic MMs are round cells, without cytoplasmic extensions (Figure 6.7), and are found in association with hepatocytes and sinusoid vessels. When comparing the same species in two environments, one in the agricultural matrix and another one in a natural environment where human impact is minimal, we observed different responses from MMs for the different species. In two anurans from the Leptodactylidae family (*P. cuvieri* and *L. fuscus*) there is an increase in the melanin area (twice as much) inside MMs. Both species are originating from an area with environmental contaminants from agricultural activities for corn and soy bean (Figure 6.8). In these environments, we observed high quantities of atrazine (5349.540 $\mu g\,L^{-1}$), besides other carbamate, organophosphate, and organochlorine pesticides, in concentrations about 10 times higher than those found in a natural environment. For both Hylidae anuran families (*H. albopunctatus* and *Scinax fuscomarginatus*), the response was the opposite, resulting in a smaller melanin area in animals from environments with agricultural activity when compared to environments without agricultural activity (Figure 6.8). For another hylid species, *D. minutus*, there were no differences in MMs, when contrasting both sampling sites. Differences in types of response from MMs collected in the same environments are related to two main aspects: (1) the pigmentation present in anurans' organs has a differential occurrence that is dependent on the species;[21] however, disregarding occurrence, these two cell types were responsive to the presence of environmental contaminants, so they are an important tool to evaluate the effects of environmental contaminants. (2) MMs' responsiveness may be related to the biology of the species: animals that live closest to the soil (*e.g.*, *P. cuvieri* and *L. fuscus*) use mainly water bodies for their vocalization; therefore, they are in contact with possible aquatic contaminants for long periods of time. *H. albopunctatus*, *S. fuscomarginatus*, and *D. minutus*, in turn, have the habit of roosting during their reproductive season, and are not in such direct contact with possible water body contaminants.

When we evaluated MMs' responses to BaP, we observed that there is a decrease in the melanin area for *P. nattereri* and *H. albopunctatus* after 3 days of exposure. For *P. cuvieri* and *L. fuscus*, the decrease happened after 7 days of exposure. For *H. albopunctatus,* after 7 days, and for *L. fuscus*, after 13 days of exposure to BaP, we observed an increase in the melanin areas in MMs (Figure 6.9). Experiments with *Xenopus tropicalis* that were exposed to BaP showed a decrease in the pigmented area of hepatic melanomacrophages,

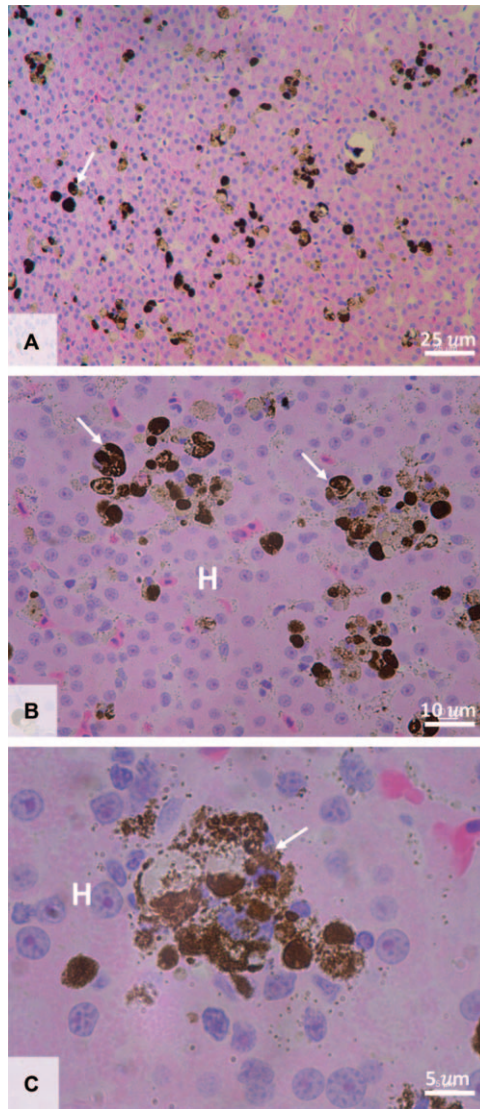

Figure 6.7 Histology of *P. cuvieri* liver (A–C) with melanomacrophages (arrows) found in hepatic tissue between the hepatocytes (H).

especially after 18 hours of exposure. Authors associate this decrease with hepatic stress and hepatocyte apoptosis.[42] Another group[43] observed degeneration and a decrease in the number of hepatic MMs in fish exposed to high levels of PAHs. They show that pollution leads to a decrease in macrophages' phagocytic activity, thus causing degeneration of MMs. Another study with PAH has also shown the same response, a decrease,[44] and recent studies with rat melanocytes have indicated that BaP is easily accumulated in tissues

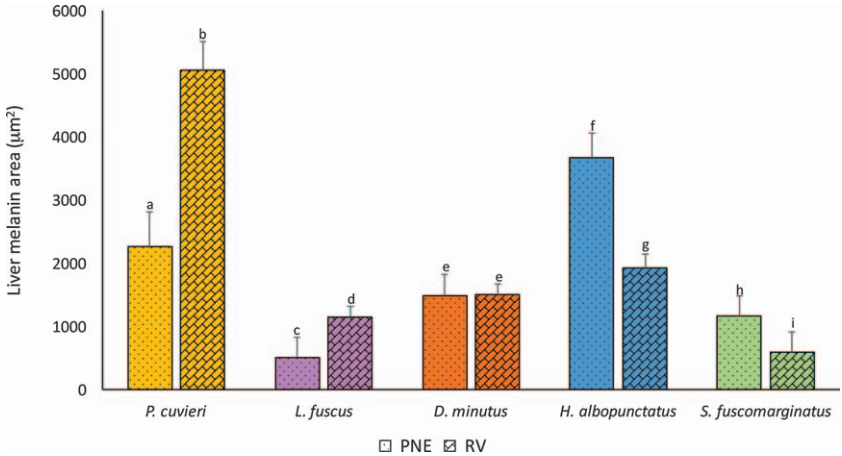

Figure 6.8 Hepatic pigmentation in *P. cuvieri, L. fuscus* (Leptodactylidae)*; D. minutus, H. albopunctatus*, and *S. fuscomarginatus* (Hylidae) collected from preserved (PNE) and from agricultural areas (RV). Observe the differences in responses among the species present in both regions. In Leptodactilidae an increased response was observed and in Hylidae decreased in agricultural areas. Different letters represent the differences among the sampling environments, for the same species.

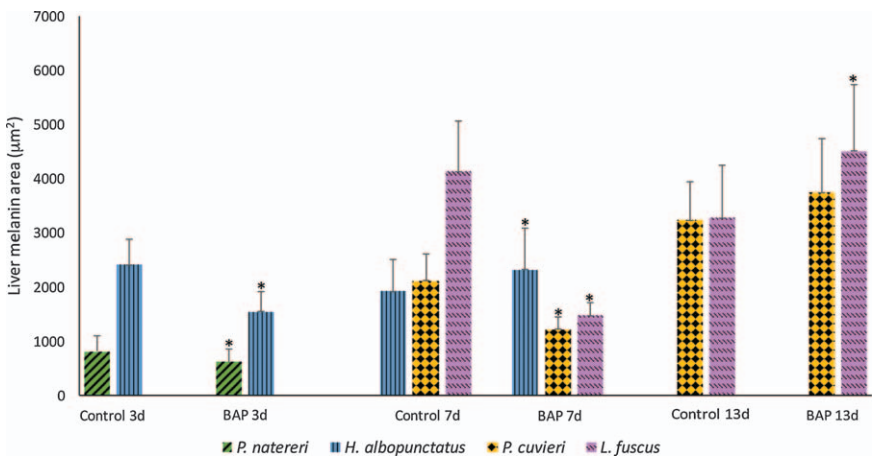

Figure 6.9 Hepatic pigmentation in *P. nattereri* exposed to 3 mg kg^{-1} benzo[alfa]-pirene (BaP); *H. albopunctatus* exposed to 7 mg kg^{-1} BaP; *P. cuvieri* and *L. fuscus* exposed to 2 mg kg^{-1} BaP. We observe a decrease in the melanin area in animals exposed to BaP after 3 and 7 days for *P. cuvieri* and *P. nattereri;* and an increase after 7 days for *H. albopunctatus* and 13 days for *L. fuscus*. Control 3d, 7d and 13d: Animals administered mineral oil for 3, 7 and 13 days, respectively. BAP 3d, 7d and 13d: Animals administered BAP for 3, 7 and 13 days. (*) represents the differences between the same experimental time.

that contain melanin; moreover, it suppresses the synthesis of the α-melanocyte-stimulating hormone (α-MSH), and decreases tyrosinase activity, two crucial elements for melanogenesis.[45] Therefore, the decrease in MMs may be associated with BaP interference in melanin production pathways. After 7 days (*H. albopunctatus*) and 13 days (*L. fuscus*) there was an increase in the melanin area in MMs. One of the functions of MMs is detoxification,[41] and one of the functions of melanin is protection against cytotoxic damage.[22] Even though BaP interferes in melanin production, when metabolized, it generates quite toxic subproducts[46] and the increase in melanin observed in MMs may be related to those functions, in order to fight toxic metabolites from the organism and reduce possible damage.

In animals from pesticide-contaminated environments, the response was the opposite of that in animals exposed to BaP, owing to this specific characteristic of the compound that is able to suppress melanogenic synthesis. In animals from environments with high human impact, MMs acted normally with the detoxification.

Recent studies with *Lithobates catesbeianus* tadpoles, an exotic animal that has been introduced in the Brazilian fauna, showed hepatotoxicity when exposed to cyclophosphamide (Figure 6.10), an antineoplastic agent.

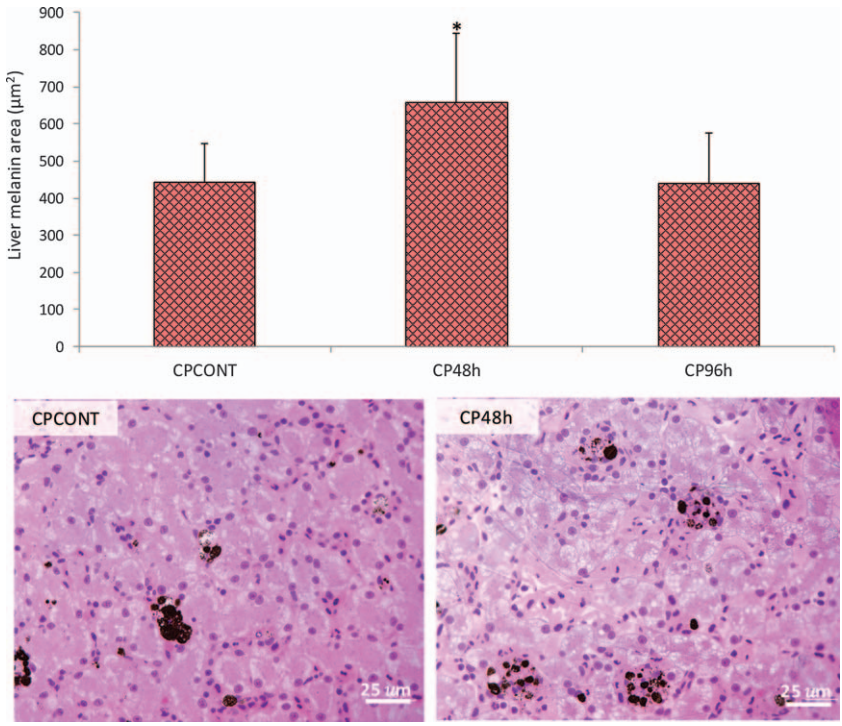

Figure 6.10 Hepatic pigmentation in tadpoles of *L. catesbeianus* exposed to 40 mg L^{-1} cyclophosphamide (CP). There was an increase in the pigmented area after 48 hours of exposure. CPCONT: animals not exposed to CP. CP 48 h and 96 h: animals exposed to CP for 48 and 96 h, respectively. (*) represents the differences between the same experimental time.

Cyclophosphamide is a drug used for chemotherapy[47] and, similarly to other medical wastes, it may contaminate the environment and affect aquatic animals. In the liver, there was an increase in the melanin area in MMs after 48 hours of exposure to the substance. Other drugs caused similar effects, for example, flutamide, an antiandrogen found in contaminated water, which caused an increase in melanin in MMs for *Rhinella schneideri* individuals.[26] Flutamide is oxidized by cytochrome P450 (CYP)[48] and transformed into reactive metabolites, a process that may lead to oxidative stress for lipid peroxidation, for the stimulation of some CYP enzymes and mitochondrial superoxide.[49,50] The process of lipid peroxidation has its peak in the production of free radicals, which may be neutralized by the melanin from MMs; that is, flutamide causes some hepatotoxic effects, thus increasing the melanin area.[26]

A synthetic sexual hormone, testosterone cypionate, is also used in medicines and may affect the *Physalaemus nattereri* anuran's liver, since an increase in the melanin area from MMs has been observed 8 days after exposure to the contaminant.[38] Another group[51] have attested that the melanogenesis in MMs is altered by the MSH hormone protein. This hormone activates the tyrosinase system, increasing the gene transcription and, in turn, increasing melanin production.

6.7 Conclusion

From the results presented, it is possible to conclude that cells containing melanin found in the organs of anurans are important for evaluating the effects of environmental contaminants, thus being highly useful in studies in ecotoxicology.

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CHAPTER 7

The Use of Terrestrial Life-stages of European Amphibians in Toxicological Studies

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7.1 Introduction

Amphibians are classified into three orders: Anura (frogs and toads), Urodela (salamanders and newts) and Gymnophiona (caecilians, only found in the Tropics). Most species have early aquatic life-stages, *e.g.* anuran larvae ('tadpoles'), while juveniles and adults are mainly terrestrial.[1] Worldwide, amphibians are showing unnatural negative population trends and unnaturally high rates of species extinction, especially in the Tropics.[2] Strong population declines were, however, also recognized in temperate regions, such as Western Europe, where dramatic declines occurred in the 1960s from which amphibian populations seem not to have recovered.[3] Several main reasons are discussed, sometimes acting in cumulative and synergistic ways, and environmental contamination—especially the high use of pesticides—is seen as one out of six supposed main factors for the global amphibian decline.[4]

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Among environmental pollutants, pesticides are of major relevance to wildlife in general and to amphibians in particular because of their intentional and widespread use (*e.g.* more than 300 000 metric tons of pesticide active ingredients are sold every year in the European Union alone[5]). Pesticide use has increased during the last few decades, mainly because human population size increased asynchronously to productive agricultural area, *i.e.* the same area produces a greater harvest, which is only possible owing to higher use of plant protection products and fertilizers.[6] Europewide, the increasing use of insecticides and fungicides in particular has consistent negative effects on overall farmland biodiversity.[7] Besides more popular animal groups that inhabit agricultural areas like birds or butterflies,[7] amphibians are also part of the farmland biodiversity; large inputs of agrochemicals have the potential to significantly impact the persistence and health of their populations.[8]

Although causal relationships between decreasing amphibian populations and increasing agrochemical use as a main factor cannot be drawn yet owing to a lack of field monitoring data, the impacts on individuals have been shown in several laboratory and field studies, but anuran larvae have mainly been used as test organisms while effects on terrestrial life-stages (*i.e.* in Europe, juveniles and adults of nearly all species of frogs, toads, newts and salamanders) apparently remain understudied.[9] For example, pesticides can suppress the immune system of adult amphibians,[10] but a clear link to increased susceptibility of diseases would require more research.[8] Most studies using terrestrial life-stages of amphibians investigated three exposure routes: (i) oral uptake of pesticides from contaminated prey (*e.g.* over-sprayed arthropods); (ii) subcutaneous/percutaneous injections of substances or injections to the dorsal lymph sac, which are far away from natural exposure scenarios; (iii) dermal absorption of substances after exposure by contact with treated surfaces or direct over-spraying (*cf.* Table 7.1). In particular, the dermal exposure route is seen as most important because amphibians absorb xenobiotics several times faster through their highly permeable skin compared to other vertebrates.[11] Terrestrial life-stages of amphibian species with annual migrations are often forced to cross agriculturally used areas at time periods when agrochemicals are applied.[12–14] Furthermore, amphibians can use these areas as summer habitats, which not only increases the risk of feeding on contaminated arthropods but also of coming into contact with contaminated soil and plant material.[15,16]

In this chapter, we consider the following two main questions concerning the potential impact of pesticide use on terrestrial life-stages of amphibians as non-target organisms:

(1) Which toxicological studies on the impact of pesticides using terrestrial life-stages of European amphibians have been conducted so far?
(2) Is the potential risk of pesticides adequately assessed for terrestrial life-stages of European amphibians using standard test organisms in current pesticide registration protocols?

7.2 Toxicological Studies on the Impact of Pesticides on Terrestrial Life-stages of European Amphibians

To obtain information on studies conducted so far using terrestrial life-stages of European amphibians, we searched Web of Science and Google Scholar by using the scientific names of all European species + "pesticide*" or "agrochemical*" as keywords. Furthermore, we employed the database ECOTOX (US Environmental Protection Agency [USEPA]) and the EFSA report by Fryday and Thompson.[17] We examined the references of the retrieved publications for further information.

For oral exposure, only four studies using European species were found. Harri *et al.*[18] found an LD_{50} value of 7.6 mg a.i. kg^{-1} for DDT using adult European common frogs (*Rana temporaria*). Cakici[19,20] examined histopathologic changes in the digestive tracts and testes following carbaryl exposure in adult Levant water frogs (*Pelophylax bedriagae*) and also found negative effects of carbaryl on the digestive tracts of adult variable green toads (*Bufotes variabilis*)[21] (Figure 7.1). These studies especially stated the risk of pesticide uptake *via* over-sprayed arthropods.

Most scientists who injected test substances (percutaneously, subcutaneously or into the dorsal lymph sac) to terrestrial life-stages considered mortality as the endpoint (Table 7.1). Survival of European common frogs (*R. temporaria*) after injection of different pesticides has been studied.[22–24] Moreover, *R. temporaria*, green toads (*Bufotes viridis*) and marsh frogs (*Pelophylax ridibundus*) varied in their LD_{50} values depending on the tested pesticide but also in a species-specific manner (*cf.* Table 7.1).[25] In Italian crested newts (*Triturus carnifex*), mortality owing to fungicide exposure (Maneb) was studied.[26] Furthermore, the carcinogenity and teratogenic effects of forelimb regeneration due to percutaneous exposure to Maneb

Figure 7.1 The variable green toad (*Bufotes variabilis*) is one out of only four European amphibian species for which data on oral exposure to pesticides is available.
Photograph taken by Burkhard Thiesmeier in Turkey.

Table 7.1 Overview of studies investigating effects of pesticides on terrestrial life-stages of European amphibians (ordered after species names).

CAS	Pesticide name	Pesticide type	Formulation	Order	Species	Life-stage	Exposure type	LD_{50} (mg a.i. kg^{-1})	Considered endpoints	Ref.
63-25-2	Carbaryl	insecticide	no	Anura	*Bufotes variabilis*	adult	oral	not stated	histopathological changes	21
55-91-4	DFP	insecticide	no	Anura	*Bufotes viridis*	adult	injection (dorsal lymph sac)	1450	survival	25
115-26-4	Dimefox	insecticide	no	Anura	*Bufotes viridis*	adult	injection (dorsal lymph sac)	1410	survival	25
311-45-5	Paraoxon	insecticide	no	Anura	*Bufotes viridis*	adult	injection (dorsal lymph sac)	188	survival	25
56-38-2	Parathion	insecticide	no	Anura	*Bufotes viridis*	adult	injection (dorsal lymph sac)	967	survival	25
107-49-3	TEPP	insecticide	no	Anura	*Bufotes viridis*	adult	injection (dorsal lymph sac)	540	survival	25
63-25-2	Carbaryl	insecticide	no	Anura	*Pelophylax bedriagae*	adult	oral	not stated	histopathological changes	19,20
4685-14-7	Paraquat	herbicide	yes	Anura	*Pelophylax* kl. *esculentus*	adult	subcutaneous injection	260	survival	34
76-44-8	Heptachlor	insecticide	no	Anura	*Pelophylax* kl. *esculentus*	larvae	not stated	not stated	toxic effects on the ventral epidermis	32
311-45-5	Paraoxon	insecticide	no	Anura	*Pelophylax ridibundus*	adult	injection (dorsal lymph sac)	91	survival	25
107-49-3	TEPP	insecticide	no	Anura	*Pelophylax ridibundus*	adult	injection (dorsal lymph sac)	34	survival	25

50-29-3	DDT	insecticide	?	Anura	*Rana temporaria*	adult	oral	7.6	survival	18
640-15-3	Ekatin	insecticide	?	Anura	*Rana temporaria*	adult	?	2480–2600	survival, changes in blood	22
122-14-5	Fenitrothion	insecticide, arcaricide	yes	Anura	*Rana temporaria*	adult	subcutaneous injection	2220–2400	survival	24
72-55-9	p,p′-DDE	insecticide	no	Anura	*Rana temporaria*	adult	injection	not stated	effects on sex hormones and retinoid homeostasis	31
22248-79-9	Tetrachlor-vinphos	insecticide	yes	Anura	*Rana temporaria*	adult	subcutaneous injection	151–192	survival	23
52-68-6	Trichlorfon	insecticide	?	Anura	*Rana temporaria*	adult	?	2040–2260	survival	23
60-51-5	Dimethoate	insecticide	yes	Anura	*Rana temporaria*	juvenile	direct over-spraying	not stated	survival	33
66441-23-4	Fenoxaprop-P-ethyl	herbicide	yes	Anura	*Rana temporaria*	juvenile	direct over-spraying	not stated	survival	33
1689-99-2	Bromoxynil-octanoate	herbicide	yes	Anura	*Rana temporaria*	juvenile	direct over-spraying	not stated	survival	33
175013-18-0	Pyraclostrobin	fungicide	yes	Anura	*Rana temporaria*	juvenile	direct over-spraying	not stated	survival	33
133-06-2	Captan	fungicide	yes	Anura	*Rana temporaria*	juvenile	direct over-spraying	not stated	survival	33
118134-30-8	Spiroxamine	fungicide	yes	Anura	*Rana temporaria*	juvenile	direct over-spraying	not stated	survival	33
12427-38-2	Maneb	fungicide	no	Urodela	*Triturus carnifex*	adult	percutaneous exposure	not stated	survival	26
12427-38-2	Maneb	fungicide	no	Urodela	*Triturus carnifex*	adult	percutaneous exposure	not stated	carcinogenity	28,29
12427-38-2	Maneb	fungicide	no	Urodela	*Triturus carnifex*	adult	percutaneous exposure	not stated	teratogenic effects on forelimb regeneration	27
12427-38-2	Maneb	fungicide	no	Urodela	*Triturus carnifex*	adult	percutaneous exposure	not stated	teratogenic effects on forelimb regeneration	30

Table 7.2 Overview of studies investigating the transport of pesticides through the skin of terrestrial life-stages of European amphibians (ordered after species names).

CAS	Pesticide name	Pesticide type	Formulation	Order	Species	Life-stage	Considered endpoints	Ref.
148-24-3	8-hydroxyquinoline	fungicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Measurement of percutaneous transport of pesticides through amphibian skin	35
1912-24-9	Atrazine	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Comparison of percutaneous transport of pesticides through amphibian and mammal skin	11
133-06-2	Captan	fungicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Measurement of percutaneous transport of pesticides through amphibian skin	35
30-54-1	DCMU	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Measurement of percutaneous transport of pesticides through amphibian skin	35
1071-83-6	Glyphosate	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Comparison of percutaneous transport of pesticides through amphibian and mammal skin	11
1071-83-6	Glyphosate	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Measurement of percutaneous transport of pesticides through amphibian skin	35
4685-14-7	Paraquat	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Comparison of percutaneous transport of pesticides through amphibian and mammal skin	11
4685-14-7	Paraquat	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Measurement of percutaneous transport of pesticides through amphibian skin	35
1918-16-7	Propachlor	herbicide	no	Anura	*Pelophylax* kl. *esculentus*	adult	Measurement of percutaneous transport of pesticides through amphibian skin	35

Figure 7.2 A calling male edible frog (*Pelophylax* kl. *esculentus*). This is a widespread species in Central Europe and has been used to study the fast dermal absorption of pesticides by amphibians.
Photograph taken by Burkhard Thiesmeier in Germany.

have been examined in *T. carnifex*.[27–30] The herbicide paraquat led to effects on antioxidant enzymes in edible frogs (*Pelophylax* kl. *esculentus*) and common frogs (*R. temporaria*) showed a significant variation in the liver retinol concentration after injection of an insecticide (p,p′-DDE).[31]

In a more realistic exposure scenario compared to injections, a few studies have also examined effects of dermal absorption of pesticides. Fenoglio *et al.*[32] investigated the toxic effects of the insecticide heptachlor on the ventral epidermis of adult edible frogs (*P.* kl. *esculentus*). They found effects on enzymatic activity, particularly of those involved in the protective response to xenobiotic injury. For seven different pesticides, Brühl *et al.*[33] studied mortality rates in juvenile *R. temporaria* after direct over-spraying. For two fungicides (a pyraclostrobin-based and a captan-based formulation) 100% mortality was observed at recommended label rates. This seems mainly be caused by the relatively fast absorption of xenobiotics through the highly permeable amphibian skin compared to other vertebrates. For example, the herbicide atrazine passes the skin of edible frogs (*P.* kl. *esculentus*) more than 300 times faster than through mammal skin[11] (*cf.* Table 7.2 and see Figure 7.2).

All in all, the impact of pesticides on terrestrial life-stages of European species must be seen as highly understudied. Only one out of 36 European urodele species and only six out of 52 European anuran species have been used as test organisms for pesticide effects so far. This is remarkable because species-specific sensitivities to pesticide injections of different European anurans were recognized[25] (*cf.* Table 7.1).

7.3 Risk Assessments for Terrestrial Life-stages of Amphibians in Pesticide Approval

Amphibians have usually been neglected in ecotoxicological studies that concern the risk assessment of pesticides.[36] The regulatory framework

generally assumes that assessments conducted on birds and mammals cover terrestrial life stages of amphibians as well. In the European Union, some changes have been recently introduced. Following the publication of regulation 1107/2009 on plant protection products, two complementary regulations published in March 2013 set out the specific data requirements for pesticide risk assessment. Regulation 283/2013, setting out the data requirements for active substances, establishes that "*available and relevant data, including data from the open literature for the active substance of concern, regarding the potential effects to birds, mammals, reptiles and amphibians shall be presented and taken into account in the risk assessment*." In practical terms, this means that published information about the effects of active substances on terrestrial amphibians must be considered as part of the risk assessment, although this is relevant only for re-registration processes because no published data are logically expected for active substances not yet on the market. Regulation 284/2013, setting out the data requirements for plant protection products (*i.e.* commercial formulations) mentions that "*where it cannot be predicted from the active substance data and, if relevant, the risk to amphibians and reptiles from plant protection products shall be addressed. The type and conditions of the studies to be provided shall be discussed with the national competent authorities*." In the EU, the responsibility of registration of active substances lies with the European Commission, while the approval of specific formulations is up to each member state. Thus, regulation 284/2013 leaves to each member state the decision on when and how risks of pesticide formulations to amphibians shall be addressed.

The incorporation of terrestrial amphibians into the new EU legislation on pesticides has generated some controversy because of the absence of tools to meet the requirements of the new regulations (*e.g.* no specific test guidelines or general risk assessment guidance document exist for amphibians).

7.3.1 Surrogate Species for Terrestrial Life-stages of Amphibians

In this section we will review how the risk assessment of pesticides is conducted with birds and mammals with the purpose of identifying to what extent terrestrial amphibians can be covered by these taxa. We will use as a reference the EU scheme for pesticide risk assessment.

The guidelines for conducting pesticide risk assessment on birds and mammals are detailed in the Guidance Document published by the European Food and Safety Authority.[37] The way that risk of pesticides on birds or mammals is assessed depends on the pesticide uptake (*e.g.* oversprayed food items). No pesticide uptake by routes other than the oral one (*e.g.* dermal, inhalatory) is considered relevant for birds and mammals. For each potential source of pesticide uptake, a tiered assessment is conducted considering both acute (LD_{50}) or long-term (no observed adverse effect level,

NOAEL) exposures, with effects on reproduction being the mechanism used to evaluate long-term toxicity. Acute oral toxicity is normally determined from the calculation of the median lethal dose (LD_{50}) after a single oral gavage. Reproductive toxicity is tested in a different way for birds and mammals. In birds, adults are fed with treated food for 10 weeks before egg laying begins, and then adult body weight and condition, as well as a series of reproductive responses (*e.g.* clutch size, eggshell thickness, hatching rate, chick survival) are recorded. In mammals, three types of reproductive toxicity tests are used: (1) a two-generation test in which both parents and the first filial generation are food-treated to further record growth, development and behavior in the first and second filial generations; (2) a prenatal development toxicity in which mothers are dosed at the zygote implantation time to monitor effects during pregnancy and on the neonates; and (3) a subchronic exposure test that can be of variable duration. In reproductive tests, a NOAEL is calculated for each recorded response, with the lowest NOAEL normally being the endpoint used for reproductive toxicity characterization.

The tiered assessment consists of different steps, at the end of which a toxicity-to-exposure ratio (TER) is calculated by diving the relevant endpoint (either LD_{50} or NOAEL) by the estimated exposure concentration. If the TER is above 10 (for acute exposure) or 5 (for long-term exposure), no further assessment is required, but if these trigger values are not reached, an assessment at the next tier level becomes necessary. However, nothing is known about environmental concentrations of pesticides in terrestrial amphibian habitats in Europe and only modelled concentrations of the active ingredients are employed.

In the first screening, an indicator species is used; this is not a real species but a model with a body size and feeding habits resulting in a higher risk than that expected for the species normally occurring in crops. The dietary exposure estimate is based on the application rate of the product and a shortcut value that depends on the concentration of the substance in the food as well as on some parameters of the model organism, such as body mass, food intake rate or fraction of the diet obtained in the treated area. In the first tier, a generic focal species is used; like the indicator species, this is not a real species either, but a model representing the real species at risk, including realistic body sizes and mixed diets. In this first tier, besides the use of a generic focal species, interception of pesticides by crops is added to the estimation of exposure to modulate the expected concentration. Finally, a refined assessment is conducted when TER trigger values are not reached during the first tier assessment either. The refinement options vary depending on the type of organism and toxicity, but generally focus on parameters to improve the realism of the exposure estimation model (*e.g.* use of real focal species, incorporation of percent of time within crop fields and diet composition values, adjustment of exposure periods and residue elements in food items), execution of field studies and development of population models.

Considering this general scheme of the risk assessment procedures relative to birds and mammals, there are two main questions to be addressed with respect to coverage of amphibian terrestrial stages:

(1) How do oral uptakes compare between terrestrial amphibians and surrogate taxa?

To understand the extent to which oral exposure of terrestrial amphibians to pesticide is covered by avian and mammalian data, we must focus on those aspects of the exposure estimation that are directly related to the organism characteristics (*i.e.* body mass, food intake rate and fraction of the diet obtained in the treated area). Modzelewski and Culley[38] studied the feeding habits of juvenile American bullfrogs (*Lithobates catesbeianus*) fed with different diets and found the maximum food intake rate to be 1.28 g day^{-1} in individuals weighing on average 14.8 g. The United States Environmental Protection Agency (USEPA) has developed a model to estimate dietary exposure to pesticides of terrestrial-phase amphibians (T-HERPS[39]). The model implements an allometric equation to estimate daily food intake (FI) in amphibians and reptiles ($FI = 0.013 \times (\text{body weight})^{0.773}$). This equation was originally based on the metabolic rate of free-living iguanids,[40] but assessment of its validity using the data collected by Modzelewski and Culley[38] revealed that the proposed equation was also suitable for amphibians. Likewise, the equation used for daily food intake by birds according to the USEPA's exposure models is $FI = 0.648 \times (\text{body weight})^{0.651}$.[41] The comparison of both equations reveals a considerably higher food intake rate in birds than in amphibians, which is something to be expected considering the high metabolic rates in endothermic animals compared to poikilothermic ones.[42] However, an especially sensitive scenario of pesticide oral uptake is that involving emerging metamorphs, which are expected to have a very active predation rate and are therefore likely to ingest contaminated prey if ponds are located inside of or adjacent to crop fields.

The question on how much of the ingested food is obtained within the treated area is difficult to estimate in wild populations, and so the percent of time within treated fields (PT) is normally used as an estimator of this parameter. As expected because of the strong seasonal, inter- and intra-specific variability in spatial behavior of the different taxa and in the different crops, the range of PT values is rather wide, and broad comparisons between amphibians and their surrogates are difficult to establish. For instance, Finch *et al.*[43] obtained calculations for a series of avian species in different crops across the United Kingdom and found that, in spring and summer months, median PT values ranged from 25 and 46% with 90th percentile values ranging from 18 to 92%.

The presence of temperate amphibians inside crop fields is also expected to be determined by the seasonal variation in activity. At the beginning of the activity period, in spring, adult individuals move

from their winter refuges to their breeding sites; these breeding migrations last for a few days at maximum,[12] during which cultivated fields can be crossed.[12–14] Food intake during the breeding season is generally constrained in amphibians,[1] so oral pesticide uptake probably has low relevance at this specific time. However, data on feeding behavior during this period are limited. Once the breeding season is finished, different movement patterns (from short- to long-distance displacements) can be observed even among individuals of the same species (*e.g.* for the natterjack toad, *Epidalea calamita*[16,44,45]), which, as in the case of birds, would expectedly result in a high variability in the PT values. Unfortunately, the information on PT values estimated from radio-tracking or other monitoring techniques in amphibians is very limited. Miaud and Sanuy[46] measured a PT of 85% for natterjack toads inhabiting an agricultural area in north-eastern Spain where the availability of crop areas was 43%, which would suggest that amphibians tend to avoid crop fields when alternative habitat patches are available at a local scale, but no overall conclusion can nevertheless be extracted from a single study.

One of the open questions is the sensitivity endpoint generated for oral exposure. We should include formulations here as well since adjuvants might also affect the toxicity of the main active molecule of a pesticide formulation. It is also unclear how high the variation between species is concerning the oral exposure pathway. The American bullfrog (*Lithobates catesbaianus*), which was force-fed with capsules filled with for example strychnine in the 1970s, might be one of the more robust amphibian species. The majority of oral exposure data for the comparison of Crane and co-workers[47] is derived from this bullfrog scenario. This industry-literature review compares a limited number of acute oral toxicity data of birds and mammals with a limited number of values for amphibians and concluded that the use of birds and mammals as surrogates for acute oral toxicity assessment in amphibians was adequate.[47] To obtain realistic sensitivity data we need to obtain data at least for a few European species that are fed with arthropods and residues of pesticide formulations. This is necessary to appropriately define a safety factor that covers the sensitivity of European amphibians.

(2) Dermal exposure in birds and mammals is assumed to be covered by oral exposure assessment, but is this also true for terrestrial amphibians?

The reason dermal exposures to pesticides are not considered in avian risk assessment is that it is assumed that the relative importance compared to the oral uptake of pesticides is so low as to make it negligible. To include more exposure routes in risk assessments is in discussion for birds (see Mineau[48] for a review), but is crucial for amphibians as pointed out below.

According to the model developed by USEPA to estimate pesticide exposure through dermal contact in birds,[49] the organism-related parameters

determining such exposure are the body weight and surface area (estimated from body weight according to the equations for each taxon available at[41]), the percent of body surface area potentially over-sprayed or in contact with treated surfaces, the dermal absorption fraction (*i.e.* fraction of the pesticide mass present on the body surface that is actually absorbed) and, for the specific case of dermal contact with treated surfaces, the rate of foliar contact (*i.e.* treated surface area that is contacted by a given surface area of the animal over the course of a time step). The rate of foliar contact is generally assumed to be a constant value derived from human exposure assessment because the fine-scale information on movement speed and frequency necessary to calculate this parameter is normally unavailable for wild specimens. Logically, the chances for dermal exposure will also be related to the presence of animals in the treated areas but, as mentioned above, it does not seem that trends in PT comparisons between birds and amphibians can be established with the currently available information. Thus, besides biometrical features, the main factors determining differences in dermal uptake of pesticides between amphibians and their surrogates are the dermal absorption fraction and the percent of body surface area potentially oversprayed or in contact with treated surfaces.

Dermal absorption fractions depend not only on the skin characteristics but also on the chemical properties of the substance. Therefore, any comparison between organisms should be made by considering the same substance, or at least substances with similar properties. We are unaware of the existence of comparisons between birds and amphibians in dermal absorption of substances, but Mineau[50] suggested that, because the skin of birds and mammals seem to work in a similar way in terms of pesticide absorption, data from mammals could be useful in estimating dermal absorption through avian skin as well. In this context, Quaranta *et al.*[11] compared the percutaneous passage of five different chemicals through the isolated, dead skins of edible frogs (*Pelophylax* kl. *esculentus*) and pigs, observing that the permeability coefficient was between 26 and 302 times higher in frogs than in pigs. Kaufmann and Dohmen[51] observed in an *in vitro* study with African clawed frog (*Xenopus laevis*) skin that, after 4 h, 71.4–87.5% and 69.0–84.8% of the applied doses of caffeine and testosterone, respectively, had diffused through the skin. These values contrast with a passage across human skin, after 24 h of exposure (*i.e.* six times longer than in frogs), of 10.9 to 46.5% of the applied dose of caffeine and 3.9 to 38.9% of the applied testosterone.[52] Compared to pig skin data obtained by Karadzovska *et al.*,[53] permeability coefficients by frog skin estimated by Kaufmann and Dohmen[51] were 148 and 95 times higher than those of pig skin.

With regard to the percent of body surface area in contact with pesticides, the USEPA avian model establishes 50% in the case of over-sprayed body surface area and 7.9% of the bird's body surface area, corresponding to the legs, for contact with treated surfaces. While the over-sprayed area is probably assumable for amphibians, the estimation of avian body surface area in contact with treated surfaces does not seem appropriate for amphibians. Tracy[54] modeled the area of the different body surfaces in northern leopard

frogs (*Lithobates pipiens*). Comparing the allometric equations obtained to estimate both the total area and the area in contact with the substrate, the percent of body surface area in contact with the substrate would vary from 6.9% in an individual weighing 10 g to 13.1% in a frog weighing 100 g. In a more recent study, Wardziak *et al.*[55] found that the average proportion of total body surface area of the palmate newt (*Lissotriton helveticus*) corresponding to ventral surfaces was approximately 18%. It must be noted that these figures, although already generally above the estimates for birds, are probably underestimating the amphibian surface area in contact with treated surfaces, as they are limited to the ventral area only, but lateral parts of the body can also be in contact with applied surfaces, especially when animals move across grassy crops. Furthermore, amphibian skin permeability is not uniform throughout the entire body, but ventral areas normally in contact with soil have high permeability to facilitate water uptake whereas dorsal surfaces are less permeable to reduce water loss.[54]

7.3.2 Indirect Effects

Additionally, current risk assessment (regardless if for mammals, birds or amphibians) does not include indirect effects of pesticides. For terrestrial life-stages of amphibians these are mainly the reduction of food or decreasing food quality. For amphibians, both could be the case since not only insecticides but also herbicides have an effect on the available food items, generally arthropods. This pathway of pesticide effects is especially crucial for juvenile life-stages of amphibians leaving the breeding ponds (metamorphs) since they need to find food rapidly in the vicinity as well as adults after spawning. A study in North East Germany could detect a decrease in the biomass of available food items after an insecticide application in June that lasted for 2 weeks (C. A. Brühl, unpubl. data). This temporal decline in food is also related to a shift in composition of food items and therefore potentially also food quality. Although this is only a first study, we assume that short-term food reductions might occur regularly after insecticide applications and affect the growth and survival of especially juvenile amphibians, whereas herbicide applications reduce insect biomass generally over a longer time period since food plants of herbivorous insects are removed. These food chain effects are so far understudied and are not included in the risk assessment approach, although the introduction of the goal of protection of 'biodiversity' as such includes all organisms and parameters such as biomass as well.

7.4 Pesticide Formulations—Toxicity in the Mix?

Pesticide products are formulated to allow the active ingredient or molecule to pass membrane barriers for enhanced uptake into a target organism, to be miscible with water by the farmer, to be stable under ambient conditions or simply to improve activity or application of a pesticide. These enhancers of effectivity are called adjuvants and pesticide products often contain more adjuvants than active molecules. A specific group of adjuvants (activator

adjuvants) are surfactants, 'surface-active agents'. Surfactants are molecules that lower the surface tension and their primary purpose is to allow for more contact between the spray droplet and the plant. The aim is that the pesticide spray droplet must be able to wet the foliage and spread out evenly over a leaf, even when it is waxy or hairy. Adjuvants themselves can be more toxic than the active molecule;[56] for instance, one group of adjuvants, the POEA (polyethoxylated tallowamine), has been proved to cause toxicity in glyphosate formulations to amphibians.[57] Other adjuvants in pesticides include solvent naphtha (petroleum distillate), which is toxic to aquatic organisms.[58] However, mostly adjuvants are declared inert.[56] The main problem for scientists working with pesticide formulations is that their identity and quantity in the pesticide product are kept confidential, so only the manufacturing industry and the authorities know the exact mixture in the formulations. Risk assessment for pesticide registration requires studies based on the active pesticide ingredient but not the mixture, which might underestimate the risk. Products are then assessed by member states for a zonal registration.

Recent dermal studies of terrestrial life-stages of amphibians already claimed that the adjuvants might play a major role in the toxicity of pesticide products to amphibians[59] and were shown to be the explanatory variable by studying two different pyraclostrobin formulations.[33] Unfortunately, even in this case the adjuvants and exact compositions of the pesticide mixtures are unknown, except for the solvent naphtha content. Both pyraclostrobin formulations contain the same amount of active chemical, but differed in the content of the main formulation additive of solvent naphtha (67% *versus* 25%). The mortality of juvenile *R. temporaria* dropped from 100% in the high naphtha product to 20% in the formulation with the lower solvent naphtha content in a laboratory over-spray scenario (an increase of toxicity owing to solvent naphta was also concluded in a recent study that investigated the effects on aquatic life-stages of *Xenopus laevis*[58]). However, other non-declared adjuvants might be responsible for the observed effect and not the naphtha. A pressing issue in understanding the toxicity of pesticides after dermal exposure of terrestrial life-stages of amphibians is the toxicity of adjuvants and the composition of pesticide formulations. It might be the case that only a few adjuvants are responsible for the observed toxicity in pesticide formulations. Screening of adjuvant toxicity is urgently required not only for amphibian but also for human risk assessment.[56]

7.5 Conclusions

With regard to the main questions of our chapter, we can make the following conclusions.

(1) ''Which toxicological studies on the impact of pesticides using terrestrial life-stages of European amphibians have been conducted so far?''

There is very little information on the impact of pesticides with regard to oral and dermal uptake in juvenile and adult European amphibians. This especially accounts for urodele species as only one out of

36 European urodeles (2.8%) has been ever used as a test organism, but also only six out of 52 European anuran species were included in specific toxicological studies. Thus, in total information on pesticide toxicity on terrestrial life-stages is available only for 8% of European amphibians. This is especially remarkable because—as for aquatic life-stages too—the few studies conducted so far show that the effect of a test substance can differ species-specifically. Furthermore, several researchers exposed amphibians by injecting the test substances and only a few studies considered more realistic oral and dermal uptake.

(2) ''Is the potential risk of pesticides adequately assessed for terrestrial life-stages of European amphibians using standard test organisms in current pesticide approval?

As pointed out above in detail, there are many crucial differences in the biology and ecology of amphibians compared to birds and mammals (which should serve as surrogates). But the most important difference is that in amphibians xenobiotics pass the highly permeable skin several times faster compared to other vertebrates and, consequently, dermal uptake of pesticides is far more relevant in amphibians than for birds and mammals. In current pesticide approval, amphibian dermal exposure is implicitly neglected due to the fact that it is not considered relevant for surrogate taxa. From this point of view (*i.e.* dermal absorption), it is very likely that birds and mammals are not adequate surrogate species for amphibians. Already 10 years ago, Smith *et al.*[60] mentioned in their review on contaminant exposure in terrestrial vertebrates that ''...most terrestrial vertebrates are probably exposed to contaminants more through the diet than any other route. Amphibians are the likely exception.'' Moreover, to this taxon-related uncertainty, we must add some general aspects that are not considered at all in ecological risk assessment, such as indirect effects and the fact that most pesticide formulations contain adjuvants, which are often more toxic compared to the active ingredient.

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CHAPTER 8

Impacts of Agriculture and Pesticides on Amphibian Terrestrial Life Stages: Potential Biomonitor/ Bioindicator Species for the Pampa Region of Argentina

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8.1 Introduction

The planet is suffering a general biodiversity crisis and many species are experiencing declines as a result of the intensive anthropogenic activities affecting ecosystems worldwide. In the midst of this global crisis, amphibians

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are the most threatened and rapidly declining vertebrate group with a rate of extinction approximately 200 times greater than background historic rates.[1,2] Factors contributing to the amphibian crisis are diverse and include habitat loss, environmental contamination, climate change and emerging infectious diseases.[3–5]

Modern agriculture is increasingly involved in the amphibian decline as agricultural expansion and intensification is causing pesticide contamination together with habitat loss and fragmentation.[6] Around the globe, freshwater systems from agricultural regions are contaminated by mixtures of diverse pesticides, which affect amphibian aquatic stages.[7–14] The terrestrial life stages of amphibians are also potentially exposed to high concentrations of pesticides when foraging and migrating within agricultural ecosystems, but this aspect of amphibian toxicology is little studied and is not normally included in risk-assessment schemes.[15]

In Argentina, the Pampa region consists of a vast grassy plain rich in lakes and ponds where agriculture and cattle ranching dominate. Over the last 40 years, the region has experienced a great expansion of cultivated surface as well as an intensification of the production through the use of fertilizers and pesticides.[16,17] The gradual transformation of the landscape, including the disappearance of many wetlands through canalization, is likely to impact the regional herpetofauna. As amphibians are key elements of food chains, whole ecosystems may eventually be altered by amphibian declines.

The biomonitoring of selected amphibian populations is a useful tool to provide information on the status and health of amphibian communities and the ecosystem in general. In the current chapter, we propose the use of five amphibian species with widespread abundance and a large South-American distribution as potential amphibian models for biomonitoring environmental quality in the Pampa region of Argentina. The characteristics and life history of the species are described, as well as current antecedents surrounding their use as bioindicators and biomonitors. Hopefully, the information contained herein will promote the development of amphibian biomonitoring programs and protocols aimed at acquiring a better knowledge of the health and status of the Pampean herpetofauna. It is essential to keep up efforts to understand the factors at play in amphibian declines so as to design adequate and effective conservation strategies.

8.2 Amphibian Diversity, Life History and Global Declines

8.2.1 Amphibian Diversity and Life History

The word ‘‘amphibian’’ originates from the Greek and means ‘‘double life’’; a reference to the fact that the typical life cycle of these animals is part aquatic and part terrestrial. Amphibians are intermediate in some ways between the fully aquatic fishes and the terrestrial amniotes. From an evolutionary point of view, amphibians have undergone a remarkable adaptive

radiation in their attainment of independence from water and colonization of land, and the living group exhibits a greater diversity of modes of life history than any other group of vertebrates.[18]

The life cycle of most amphibians includes laying permeable eggs in water, which is followed by an aquatic larval stage, a period of metamorphosis from larva to juvenile, and an adult stage that can occur in both aquatic and terrestrial habitats. Although this classical statement is true in many cases, it nevertheless represents a coarse oversimplification of the reality as amphibian life histories are varied and complex, and many are still not understood. From a taxonomic perspective, the class "*Amphibia*" is divided into three orders: *Gymnophiona* (caecilians), *Urodela* (salamanders), and *Anura* (frogs and toads). The order *Anura* is by far the largest and most varied with 5602 species, compared to 174 and 571 species for the Gymnophiona and Urodela orders, respectively.[19] The word Anura means "without tail" in Greek, in reference to the fact that the order is composed of frogs and toads, which are tailless after metamorphosis. Anuran live everywhere except where restricted by cold temperatures or extremely dry conditions. Anurans live in the water, on the ground, underground and in the trees. Their body length ranges between 13 mm and 30 cm, they have long strong back legs well adapted for jumping and the males of most species call to attract females for mating.[19]

Regardless of their great diversity, all amphibians share certain physiological characteristics that together set them apart from other terrestrial vertebrates: (1) They have a scale-less permeable skin, which allows for rapid passage of both water and respiratory gases. (2) They are ectothermic, which means they are incapable of generating their own body heat and depend on the sun to raise their body temperatures. (3) They are somewhat dependent on water for reproduction because the egg is never protected by a hard shell and therefore loses and gains water across the egg membrane very rapidly.[20]

8.2.2 Amphibian Declines

The planet is currently suffering a biodiversity crisis as extinction rates are close to a thousand times higher than background levels estimated from fossil records.[21] The escalating extinction crisis led scientists to claim that human-induced changes to the Earth's biosphere have driven the earth into its sixth mass extinction.[22–24] For many, the biodiversity crisis is evidence that the diversity of nature cannot support the current pressure that humanity is placing on the planet. The sum of human-induced changes to the Earth's biosphere is indeed considerable and is expressed in the recent fossil and sedimentological record.[25] Only one-quarter of the Earth's ice-free surface now represents natural wilderness,[26] and occurrences such as climate change and distribution of pollutants prove that there is no place on Earth that has not been altered by humans.[24]

Amphibians constitute the prime example of the current widespread biodiversity crisis as they are the most threatened and rapidly declining vertebrate group.[3,27] In its first global assessment of amphibian species, the

International Union for Conservation of Nature (IUCN) determined that 32% of amphibian species are threatened and that 43% are in decline.[27] In parallel, Roelants *et al.*[1] calculated that the current rate of extinction of amphibian species is 200 times greater than the historic rate.

It has now been 26 years since the problem of global amphibian decline became widely recognized.[28] In this period, scientists have identified six major threats to amphibians: habitat loss and fragmentation, commercial over-exploitation, introduced species, environmental contaminants, global climate change, and emerging infectious diseases, especially the chytrid fungus, *Batrachochytrium dendrobatidis*.[19] However, efforts to link specific threats to the species they affect or to use the scientific data on a large scale to discern the causes of the disappearance have generally failed. A recent study conducted at the national scale in the United States has found that the presence and intensity of the four main threats, human influence, disease, pesticide application, and climate change, vary substantially across the country and that the causes of the declines are more variable, and more locally driven, than had been assumed.[29]

Amphibian declines may ultimately result in secondary impacts on ecosystems as amphibians play a key role in energy flow and nutrient cycling as they are both predator and prey. For example, tadpoles reduce the rate of natural eutrophication by eating huge amounts of algae. As ectotherms, amphibians are efficient at converting food into growth and reproduction. Indeed, amphibians convert about 50% of their energy gained from food into new tissue, which is transferred to the next level in the food chain.[19]

8.3 The Pampa Region of Argentina

8.3.1 Location, Geography and Characteristics

In Argentina, the region known as ''the Pampa'' consists of a vast grassy plain of about 500 000 km^2 that covers most of the central sector of the country. The Argentine Pampa is part of a vast continental plain known as Pampasia that separates the ancient shields of Guyana–Brasilia and the Andean system, and includes the great regions of the Amazonas and the Chaco. The Pampa region is located between the 31° and 39° south parallels of latitude and between the 57° and 65° west meridians of longitude (Figure 8.1). The eastern limit is constituted by the rivers Uruguay and La Plata, and the Atlantic Ocean.[30] It includes the totality of the provinces of Buenos Aires and Entre Ríos, the center and south of the province of Santa Fe, the center and southeast of the province of Cordoba, and the northeast of La Pampa Province. The landscape is flat or slightly undulating and the native vegetation is composed of small bushes, grass and gramineous. Climate is temperate within the region: mean precipitations gradually decrease from 1100 mm to 600 mm per year from east to west, and mean annual temperatures gradually increase from 14 to 19 °C from south to north.[31] Agriculture and cattle ranching occupy most of the territory as the temperate climate and fertile deep soil have favored the establishment of a thriving farming economy.

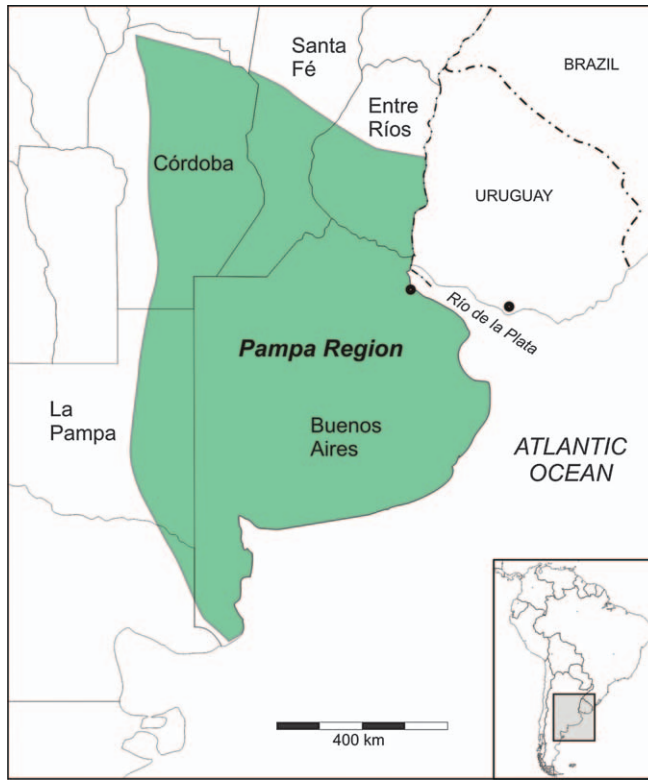

Figure 8.1 Location of the Pampa region within Argentina.

A distinctive characteristic of the Pampa region is the abundance and variety of wetlands. The Pampean plain is spattered with ponds and lakes of soft or brackish water. In the province of Buenos Aires alone, there are 1429 lakes or ponds with a length equal or superior to 500 m.[32] The lakes and ponds of the Pampas are unique habitats, which are typical and exclusive to the region. They may be defined as permanent or temporary water bodies that contain water of medium to high salinity. They are generally not very deep and they do not present a defined thermal cycle or a permanent stratification. They can be entirely colonized by vegetation as light can penetrate the entire water column owing to its low depth.[33,34] Given the flat relief of the region, vertical water movements prevail, and so the main water fluxes are through rainfall, evaporation and infiltration.[35]

8.3.2 Evolution of Agricultural Practices and Environmental Impacts

Although the argentine Pampa has a centuries-long history of farming, the agriculture of the region underwent dramatic transformations over the last 40 years, which led to a complete modification of the farming systems and agricultural practices. The process, which began in the mid-1970s, is the

result of a conjunction of different pressures, such as the adoption of new technologies, economic concentrations, the increase in the production scale, new organizational forms, and the orientation and dependence on the exterior market.[36] Over this period, the Pampa has gone through an accelerated process of agriculturization where farming activities passed from a mixture of cattle ranching and grain production to monocultures that first involved corn, sunflower, and wheat but that later focused mainly on soybean.[37] This is how many subregions that were formerly devoted uniquely to cattle ranching or to a mixture of both cattle ranching and grain production have nowadays become exclusively dedicated to grain production.[16]

The transformation of the Pampa also implied a great expansion of the cultivated surface as well as an intensification of the production, which was predominantly based on the use of high-yielding crop varieties, chemical fertilizers and pesticides, and mechanization.[16,17] In the last 40 years, the total planted surface in Argentina more than doubled from 14 to 31 million hectares, and the production of wheat, corn, sunflower, soybean, and sorghum substantially increased from 21 to 96 million tons.[38] As mentioned above, the changes were not equal for all crops and majorly focused on soybean, which was the first genetically modified (GM) crop to be commercialized in Argentina.[39] The sale of glyphosate-resistant soybean was first approved in 1996 and, since then, the total planted area grew from 6 to 20 million hectares.[38] Nowadays, numerous varieties of soybean and corn genetically modified to be resistant to herbicides or to insects are being commercialized in the country. Argentina is now the world's third largest producer of GM crops, following the United States and Brazil in terms of production.[40]

The main genetic trait incorporated in crops is the tolerance to the broad-spectrum herbicide glyphosate, which allows producers to apply this herbicide to control weeds without harming crops.[41] Glyphosate-resistant soybean, corn, cotton and canola constitute the four principal GM crops used in the United States, Brazil and Argentina.[40] As a consequence of their wide use on transgenic crops, glyphosate-based herbicides are currently the world's bestselling herbicides.[41] In Argentina, the extensive planting of GM crops generated a more than 160% increase in the annual use of glyphosate: from 1 to 162 million liters between 1994 and 2011.[42]

The use of glyphosate-resistant GM crops is closely linked to the wide adoption of no-till agriculture, which is now employed in nearly 80% of the planted superficies in the region.[43] No-till is an agricultural production practice where the soil is left undisturbed from harvest to seeding and from seeding to harvest. Weed control relies on herbicides applied pre-plant, pre-emerge or post-emerge, a modern practice made possible by the advent of herbicide-resistant GM crops.[44] Although no-till production can provide soil-quality and conservation benefits, its dependence on herbicides and the overreliance on glyphosate now threaten its sustainability.[45] Indeed, this single-tactic approach to weed management has resulted in unintended, but not unexpected, problems: a dramatic rise in the number and extent of weed

species resistant to glyphosate[46] and a concomitant decline in the effectiveness of glyphosate as a weed management tool.[47,48] The number and extent of weed species resistant to glyphosate has increased rapidly since 1996, with 21 species now confirmed globally.[46]

In response to the outbreak of glyphosate-resistant weeds, biotechnology industries have developed crops that are genetically modified to have combined resistance to glyphosate and synthetic auxin herbicides. However, concerns exist that if the rate of adoption of this technology follows the general trajectory of glyphosate-resistant crops, the result could be a profound increase in the total amount of herbicide applied to farmland.[45] In fact, although the issue is highly debated, claims exists that the problem of pesticide-resistant weeds have already resulted in a global increase in pesticide use,[49,50] in contrast to the often-repeated statement that today's genetically engineered crops have reduced pesticide use.[40,51,52] In the Pampa, the principal insecticides used in grain crops include the pyrethroids (cypermethrin and lambda-cyhalothrin), the organophosphate chlorpyrifos and the neonicotinoid thiamethoxam. The most used fungicides are carbendazim and tebuconazol, while the principal herbicides, aside from glyphosate, include atrazine, acetochlor and metolachlor, 2,4-D, chlorimuron and metsulfuron.[42] These pesticides can be applied alone or, more commonly, in various combinations.[53–55]

8.4 Agriculture and Amphibian Declines: The Need for Biomonitoring

8.4.1 Agriculture and Amphibian Declines

Although many factors contribute to amphibian declines, either singly or in combination, biologists worldwide agree that habitat loss, alteration, and fragmentation play a major role in the problem.[56–59] Over the last 100 years, agricultural expansion and intensification has been the main driver of the conversion of natural habitats. During this period, the agricultural area has become one of the largest terrestrial biomes on Earth, occupying more than 40% of the land surface.[60,61] At the same time, half of the world's wetlands have been lost,[62,63] mainly owing to drainage and replacement with agricultural land.[64]

Agriculture-mediated habitat fragmentation can render species prone to local extinctions and declines as a result of diminished population sizes and reduced colonization and immigration rates.[65,66] Additionally, ponds and wetlands are essential for the breeding as well as the embryonic and larval development of many amphibians. Hence, amphibians may be particularly susceptible to landscape alterations, such as the loss and increased isolation of important habitat types caused by agricultural intensification, as their habitat requirements are multiple and complex.[59,67]

In addition to habitat loss and fragmentation, modern agriculture is likely affecting amphibians through the pesticides and other agricultural chemicals it releases into the agricultural ecosystems in greater varieties and

combinations, and to a greater extent, than ever before.[6,68] Pesticides are toxic chemicals designed to be deliberately released into the environment. Because of the risks associated with the use of pesticides, all countries have established laws and regulations to control the production, trade and use of pesticide products. Crop protection products applied on the fields may enter ponds and wetlands through drift, accidental overspray or runoff.[69,70] Extensive water quality monitoring programs from agricultural regions of North America indicate that diverse pesticide contaminants are often present at low concentrations throughout the year and that herbicides are commonly detected in 70 to 90% of the samples.[8,9] Based on laboratory and mesocosm experiments, this widespread contamination is likely affecting embryos and larval stages,[6,67] for example through endocrine disruption by atrazine[71,72] and increased mortality from environmentally relevant glyphosate exposure.[73]

While ponds and wetlands only receive the drift and runoff of pesticide applications, terrestrial habitats like the fields themselves, on the other hand, receive intentional pesticide applications at full rates.[15] Terrestrial life stages of amphibians such as juvenile and adult frogs, toads and newts foraging and migrating within agricultural ecosystems are potentially exposed to these high concentrations of pesticides. Although potentially harmful for amphibian populations, impacts on terrestrial life-stages remain little studied and represent one of the most important knowledge gaps that remains within the field of amphibian toxicology.[74] As opposed to birds and mammals, for amphibians to date no specific risk assessment is required for the registration of a new pesticide product. This situation is worrisome, especially in view of a recent study, which showed that mortality of juvenile frogs in an agricultural overspray scenario ranged from 100% after 1 hour to 40% after 7 days at the recommended label rate of the registered products.[61]

8.4.2 Amphibians as Bioindicators and Biomonitors

For many, current global amphibian declines serve as a warning that we are in a period of significant environmental degradation as amphibians are considered one of nature's best indicators of overall environmental health. Indeed, amphibians are considered uniquely sensitive to man-made changes in the environment because their porous skin is vulnerable to waterborne toxins and infections, and their reliance on two habitats (water and land) means they cannot survive properly without both.[20] In addition, embryos and larvae of amphibians with external fertilization and development are susceptible to environmental pollutants owing to direct exposure.

Because they exhibit an early response to environmental stress and degradation, amphibians can be useful for biomonitoring. Biological monitoring, or biomonitoring, is the use of bioindicator species or biological responses (biomonitors) to assess changes in the environment, generally changes owing to anthropogenic causes. Bioindicators are organisms (or communities of organisms) that provide **qualitative** information on the quality of the environment by their presence or absence, or through the

display of other typical symptoms; behavioral or physiological. On the other hand, biomonitors are organisms that provide **quantitative** information on the level of environmental contamination, through measurable or physiological or biochemical changes (called biomarkers). Biomonitoring is a valuable assessment tool that is being increasingly utilized in environmental quality monitoring programs of all types.

Biomonitoring schemes involving amphibians can be designed to aim at one or both of two different objectives: (1) to inform on the status and health of the amphibian community, or (2) to assess the health of the aquatic and/or terrestrial component of an ecosystem. An important aspect to take into account when biomonitoring with amphibians is the diversity of life histories that exist within the amphibian world. It is, indeed, important to consider species inhabiting all possible compartments (land, water, vegetation), as the pollutant exposure experienced by each one of them may be dramatically different.

8.4.3 Suggested Amphibian Model Species for Biomonitoring the Pampa Region of Argentina

In Argentina, the amphibian fauna consists of 176 species divided into two orders, 13 families and 40 genera. Most amphibians are anurans as the *Urodela* order is not represented in the country, and the *Gymnophiona* order counts only three genera.[75] As specifically regards the Pampean region, it counts 26 species of amphibians from seven families, as presented in Table 8.1. Most species are anurans and only one species belongs to the Cecilidae family.

The species we propose for biomonitoring in the Pampa region of Argentina are abundant species that present a wide distribution in the neotropics, which means they can be found and utilized in most of the Pampa region and in other South American countries. With regards to their conservation status, all proposed species are considered of ''least concern'' or ''not threatened'' according to both the IUCN[76–81] and the Argentine Herpetological Association classifications.[82] When considered as a group, the proposed model species inhabit and reproduce in all components of the ecosystems (land, water, vegetation), so all life strategies are encompassed (Table 8.2).

According to Holt and Miller,[83] bioindicator and biomonitor species should be moderately tolerant to environmental variability. Therefore, rare species with narrow tolerances are too sensitive to environmental change, or too infrequently encountered, to be used, whereas species with very broad tolerances are too insensitive to environmental change. Because the proposed model species for the Pampa region are widely distributed, they do not qualify as rare or too sensitive. What remains to be proven through future field studies is whether the proposed species do present enough sensibility to react to environmental changes before the rest of the community is affected.

Table 8.1 List of amphibian species inhabiting the Pampa region of Argentina.[a]

	Red List category status	Population trend
Order Gymnophiona		
Familia Caeciliidae		
Chthonerpeton indistinctum	LC	Unknown
Order Anura		
Familia Bufonidae		
Rhinella arenarum	LC	Stable
Rhinella dorbignyi	LC	Stable
Rhinella fernandezae	LC	Stable
Family Ceratophrydae		
Ceratophrys ornata	NT	Decreasing
Family Cycloramphidae		
Odontophrynus americanus	LC	Stable
Odontophrynus occidentalis	LC	Unknown
Family Hylidae		
Argenteohyla siemersi siemersi	EN B2 ab[b]	Decreasing
Dendropsophus nanus	LC	Stable
Dendropsophus sanborni	LC	Stable
Hypsiboas pulchellus	LC	Stable
Lysapsus limellum	LC	Stable
Pseudis minuta	LC	stable
Scinax berthae	LC	Stable
Scinax granulatus	LC	Stable
Scinax nasicus	LC	Stable
Family Leiuperidae		
Physalaemus biligonigerus	LC	Stable
Physalaemus fernandezae	LC	Unknown
Physalaemus henselii	LC	Stable
Physalaemus riograndensis	LC	Stable
Pseudopaludicola falcipes	LC	Stable
Family Leptodactylidae		
Leptodactylus gracilis	LC	Stable
Leptodactylus latinasus	LC	Stable
Leptodactylus mystacinus	LC	Stable
Leptodactylus latrans	LC	Stable
Family Microhylidae		
Elachistocleis bicolor	LC	Stable

[a]LC = Least Concern, NT = Near Threatened, EN = Endangered.
[b]B2 ab Area of occupancy estimated to be less than 500 km^2, severely fragmented or known to exist at no more than five locations; continuing decline, observed, inferred or projected, in area, extent and/or quality of habitat.

The group of species we propose for biomonitoring in the Pampa region of Argentina are: *Leptodactylus latinasus*, *L. latrans*, *Hypsiboas pulchellus*, and *Rhinella fernandezae* in conjunction with *R. dorbignyi*, and *R. arenarum*.

Table 8.2 Habitat and reproduction site of proposed model amphibian species for biomonitoring in the Pampa region of Argentina.

	Habitat	Reproduction
Order Anura		
Familia Bufonidae		
Rhinella arenarum	Land	Water
Rhinella dorbignyi	Land (cave-dwelling)	Water
Rhinella fernandezae	Land (cave-dwelling)	Water
Family Hylidae		
Hypsiboas pulchellus	Vegetation	Water
Family Leptodactylidae		
Leptodactylus latinasus	Land (cave-dwelling)	Land
Leptodactylus latrans	Land/water	Water

The characteristics and life histories of these species are described in the following section and compared in Table 8.2. *R. fernandezae* and *R. dorbignyi* are considered as alternative species because they are very similar in most aspects and their distributions are often exclusive. The decision to use *R. fernandezae* or *R. dorbignyi*, will therefore be based on the geographic location where biomonitoring is to take place.

8.5 Description and Life Histories of Model Amphibian Species for the Pampa Region of Argentina

8.5.1 *Leptodactylus latinasus* (Jiménez de la Espada, 1875)

Common Names: Oven Frog, Urnero, Rana Piadora.

Distribution: Northeastern Argentina, all of Uruguay, State of Rio Grande do Sul in Brazil, most of Paraguay, southeastern Bolivia.

Size: Males 27–38 mm, females 29–37 mm.

Description: A small frog with a pointed snout. Back brownish, densely granulated, with irregular dark markings, a triangular dark inter-ocular mark, and an inter-scapular diamond shaped reddish mark that is sometimes difficult to observe. Whitish glandular chain on the flanks. Pale whitish underside. Round transparent tympanum. Extremities with wide dark transversal bands. Dark upper lip strip. Males have dark bilateral gular markings on the vocal sac (Figure 8.2).

Habitat: Terrestrial species found in grassland habitats. A cave-dweller, it hides under tree trunks, stones and in burrows or crevices. Prefers patches of mud, ground with crevices, and short grass with mud.

Reproduction: Throughout spring and summer: from October to February. Males call from within 10 centimeter deep burrows that they dig near water or in crevices and depressions that will eventually be flooded. During the

Figure 8.2 *Leptodactylus latinasus*.

amplexus, males use hind legs to froth up an albumin substance produced by the female to make a foam nest for the eggs. Eggs are pale yellow. Eggs and larvae develop in the burrow, within the foam nest, and emerge when expulsed by a rain shower.

Alimentation: Feeds on spiders and insect larvae as well as on isopterans, coleopterans and other small insects. A generalist with a foraging strategy that can be considered intermediate of a sit-and-wait and an actively foraging predator. It has a wide spatial niche and consumes a great diversity of prey.[84]

Conservation Status: Listed as **Least Concern** in view of its wide distribution, tolerance of a degree of habitat modification and presumed large population.[76]

8.5.2 *Leptodactylus latrans* (Steffen, 1815)

(formerly known as L. ocellatus)[85]

Common Names: Criolla Frog, Rana Común.

Distribution: Widely distributed over South America east of the Andes from Venezuela to Argentina: North of Argentina, all of Uruguay, most of Paraguay and Brazil, northeast of Bolivia and Colombia and most of Venezuela.

Size: Males 140 mm, females 120 mm.

Description: A large long-legged anuran. Eight longitudinal folds on the back. Backside greenish or yellow-brown with notable longitudinal lines of a clearer color. Triangular dark inter-ocular mark. Clearly notable tympanum. Whitish underside. It is one of the few amphibians in which the males are clearly larger than the females. Mature males typically have large well developed arms and two conical spines on the first finger of the hand. The vocal sac is internal in males (Figure 8.3).

Habitat: Occurs in various habitats, including savannahs, grasslands, open habitats in dry areas, forest edge, and along riverbanks in humid tropical forests. These semi-aquatic frogs are both diurnal and nocturnal. They are frequently found resting at the margin of ponds or shallow bodies of waters and jump into the water if disturbed.

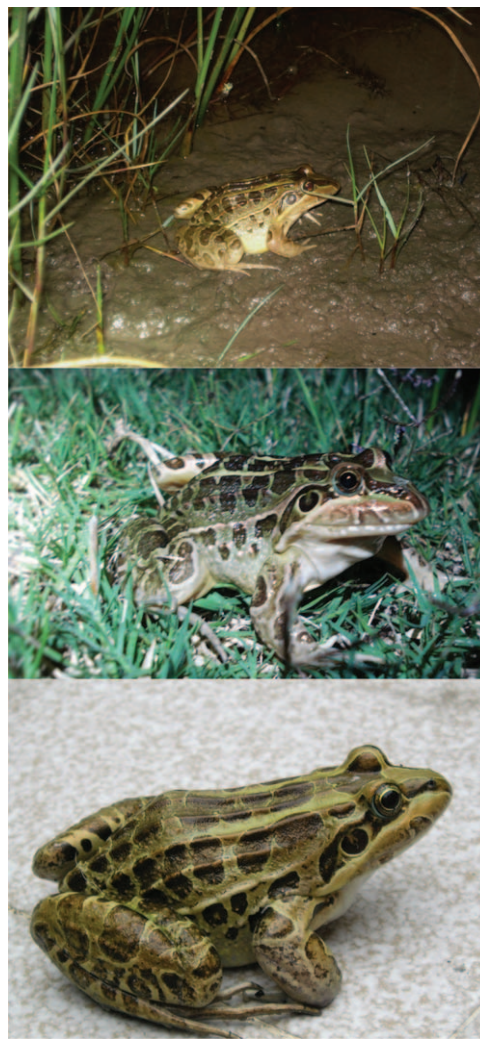

Figure 8.3 *Leptodactylus latrans*.

Reproduction: Throughout spring and summer: from September to February. Males hide in aquatic vegetation and call with short monotonous notes at low pitch. Calls are louder and more frequent before rain, as the eggs are usually laid into seasonal ponds. During breeding, the female secretes an albuminous substance that both parents beat with their feet to form a floating foamy nest of 10 to 25 cm of diameter where black-pigmented eggs are deposited. The female protects its eggs until hatching by remaining in the middle of the nest, and later on keeps on defending her tadpoles by attacking potential aggressors.

Alimentation: These are active and vigorous frogs, and owing to their wide trophic range they have been classified as generalist consumers.[86] They are voracious as a predator, which makes them highly competitive species. Their diet consists of insects and their larvae, beetles, arachnids, lepidopters, ants, annelids and, with lower frequency, other smaller anurans.[86,87]

Conservation Status: Listed as **Least Concern** in view of its wide distribution, tolerance of a broad range of habitats, presumed large population.[77]

8.5.3 *Hypsiboas pulchellus* (Duméril and Bibron, 1841)

(formerly known as Hyla pulchella)

Common Names: Montevideo Treefrog, Rana de Zarzal, Rana Trepadora Común.

Distribution: Northeast Argentina, all Uruguay, states of Rio Grande do Sul and Santa Catalina in Brazil, and southern Paraguay.

Size: Males 32–46 mm, females 41–48 mm.

Description: Possesses a capacity for camouflage, its dorsal coloration changing from light-brown/beige to green, immaculate or scattered with dilute dark blotches, depending on the surface where the animal was resting. Head as wide as long. Big eyes and small but notable tympanum. Horizontal pupils. Fingers with sucker discs that will facilitate climbing. A white line runs from the eye backwards and is shadowed below by a dark line; both extend onto the flanks. Granulated belly light in color. Yellowish vocal sac. Mature females are slightly larger than males (Figure 8.4).

Habitat: Spends most of the day concealed amongst the vegetation, clinging on to leaves or branches. Can be found in a variety of habitats including wetlands, farmlands and semi-urban settings.

Reproduction: In the Pampean region of Argentina, reproductive activity is most intense in three distinct moments of the year: August–September, November–December and March–April.[88] The species presents two different calls: the first one is brief and can be heard before or after a rainfall or at sunrise and sunset, while the second call is more intense and is associated with reproduction.[88] Males call from near the water, over reeds and other vegetation. Dark eggs in transparent jelly, on the bottom or stuck to the vegetation. Tadpoles are large and develop slowly.

Alimentation: Consumes beetles, spiders, flies and mosquitoes. While in the coldest months, most species go into hibernation and decrease the activity of their populations drastically, *H. pulchellus* continues feeding even

Figure 8.4 *Hypsiboas pulchellus*.

at low temperatures to provide the extra energy necessary to allow courtship in males.[89]

Conservation Status: Listed as **Least Concern** in view of its wide distribution and presumed large population.[78]

8.5.4 *Rhinella dorbignyi* (Dumeril and Bibron, 1841) and *Rhinella fernandezae* (Gallardo, 1957)

(formerly known as Bufo dorbignyi and Bufo fernandezae)

Common Names: Bella Vista Toad, Sapito común, Sapito de Jardín, Sapito de Panza Amarilla, Sapito Cavador o Sapito de las Cuevas.

Distribution:

R. dorbignyi: North eastern Argentina (Buenos Aires), Uruguay (Artigas, Canelones, Cerro Largo, Lavalleja, Maldonado, Rivera, Rocha, Tacuarembó), and southern Brazil (Rio Grande do Sul).

R. fernandezae: North eastern Argentina (Buenos Aires, Córdoba, Corrientes, Entre Ríos, La Pampa, Santa Fe), southern Paraguay, Uruguay (Canelones, Colonia, Montevideo, Río Negro, San José) and southern Brazil (Rio Grande do Sul).

Size:

R. dorbignyi: Males 38–67 mm, females 44–76 mm.

R. fernandezae: Males 36–64 mm, females 42–69 mm.

Description: *R. dorbignyi* and *R. fernandezae* are two very similar sympatric species, whose main difference resides in the pattern of the cephalic crests.[90]

R. dorbignyi presents a reduced postorbital crest, supraorbital crest higher and bulkier, forming a straight line with canthal and supratympanic crests, infraorbital crest absent or reduced and short.

Figure 8.5 *Rhinella fernandezae*.

R. fernandezae presents a more developed postorbital crest, supraorbital crest lower, infraorbital crest is always present and extending beyond postorbital crest.

The rest of the description is similar for both species: Broad head, short snout. Small tympanum. Small parotid glands, prominent eyes with horizontal pupils. Short hind legs. Greenish or yellowish brown dorsum with dark diffuse markings and a yellowish mid-dorsal line from the snout to the vent. The underside is yellowish. Males have dark vocal sac. Females are larger than males (Figure 8.5).

Habitat: Found terrestrially in grasslands. They are cave-dwelling species. When not reproducing, they remain in vertical burrows 20 cm deep that they dig using their hind legs.

Reproduction: Throughout spring and summer, following large precipitations: from September to March. Call is a long, loud and steady-tempered buzz, sounded both day and night. Eggs are laid in gelatin-like spiral threads laid in submerged grass or at the bottom of temporary puddles.

Alimentation: Ant specialists but also feed on beetles and spiders.

Conservation Status: Listed as **Least Concern** in view of its wide distribution, tolerance of a broad range of habitats and presumed large population.[79,80]

8.5.5 *Rhinella arenarum* (Hensel, 1867)

(formerly known as Bufo arenarum)

Common Names: Common Toad, Sapo Común.

Distribution: Northern half of Argentina, Uruguay, southwestern Brazil, parts of Paraguay and Bolivia.

Size: Males 88–108 mm, females 93–108 mm.

Description: Concave head. Prominent eyes with horizontal pupil. Visible round tympanum. Elongated parotid glands. Females larger than males. Males strong limbed, greenish back. Yellowish vocal sac with darker granulations. In males, fingers 1, 2 and 3 have black callosities in breeding season. Females dorsally olive green with grey blotch or vice versa. White underside (Figure 8.6).

Habitat: Found in a variety of locations, from dry to humid habitats and in suburban settings.

Reproduction: Throughout spring and summer, following large precipitations: from September to March. Males call at night from the water of

Figure 8.6 *Rhinella arenarum*.

temporary water bodies or on the exposed edge of the water. Long loud even-pitched call, even in daylight during spawning season. Oviposition occurs in long strings of over 30 000 eggs at a time. Tadpole stage lasts about 45 days.

Alimentation: Feeds on a variety of insects, worms, spiders and small vertebrates.

Conservation Status: Listed as **Least Concern** in view of its wide distribution and presumed large population.[81]

8.6 Previous Biomonitoring Studies Conducted with Proposed Amphibian Model Species

8.6.1 Studies Using Model Species as Bioindicators

In an agricultural landscape from central-eastern Argentina, Sánchez *et al.*[91] determined that agricultural land use can alter the structure of anuran assemblages (which included *H. pulchellus*, *L. latinasus*, *L. latrans*, *R. arenarum* and *R. fernandezae*) and has an effect on their breeding ecology. Attademo *et al.*[92] examined the diversity of amphibians in soybean fields of the Córdoba and Entre Ríos provinces of Argentina and concluded that soybean fields likely support large anuran populations of species such as *R. arenarum* and *L. latinasus*, and the semiaquatic *L. latrans*. For their part, Peltzer *et al.*[93] assessed the diversity and composition of anuran amphibians in 31 agricultural ponds in mid-western Entre Ríos Province, Argentina. The species present in the control site and in the agricultural ponds were *R. arenarum*, *R. fernandezae*, *L. latinasus*, *L. latrans*, and *H. pulchellus*, among others. They found that an increase in the area and depth of the ponds, width of the field margins and diversity of associated vegetation best explained the increase in diversity and composition of anuran amphibians in agricultural ponds.

Maragno *et al.*[94] studied the role of phytophysiognomies and seasonality on the structure of ground-dwelling anuran populations in the Pampa from southern Brazil. Considering that habitat use by amphibians is related both to climate and environmental features, they tested the hypothesis that anuran assemblages found in different phytophysiognomies and in different seasons vary in structure. The study took place in three phytophysiognomies: grassland, ecotone grassland/forest; and forest; and the seasonality factor was created by grouping months into warm and cold seasons. Sixteen species were found and the assemblages were influenced both by phytophysiognomies and climatic seasonality. Heterogeneous phytophysiognomies are important for maintaining abundance and constancy of populations of anurans.[94]

Seasonal variation in abundance of a whole assemblage (adults and juveniles), coexistence patterns and phenology were analyzed in lowland river floodplain ponds.[95] The authors registered 16 anuran species, including *H. pulchellus*, *L. latrans* and *R. fernandezae*. Reproduction and recruitment were adjusted to coincide with favorable environmental conditions and resource availability during the warm and rainy season, with flood pulses

playing an important role among the determinants of amphibian activity in the studied floodplains.[95]

Recently, Suarez *et al.*[96] evaluated the anuran response to landscape composition and configuration in two landscapes of east-central Argentina with different degrees of agriculturalization through call surveys. Anuran richness (including *H. pulchellus*, *L. latinasus*, and *R. fernandezae*) was lower in the landscape with a greater level of agriculturalization and with reduced amount of forest cover and stream length. They concluded that anurans within agricultural landscapes of east-central Argentina are responding to landscape structure. Responses varied depending on species and study scale. Life-history traits contributed to response differences.

8.6.2 Studies Using Model Species as Biomonitors

8.6.2.1 Studies with Leptodactylus latinasus

The helminth community structure in *L. latinasus* was studied in Corrientes, Argentina.[97] The helminth component community of this frog population consisted of 17 species and the most infected organs were kidneys, small intestine, large intestine and pharyngeal zone.

Age, body size and reproductive potential were compared between *L. latinasus* from a field planted with soybean and from a nature reserve. Individuals from the soybean field were significantly younger and smaller than those from the nature reserve. None of the frogs sampled in the soybean field had reached sexual maturity whereas 33% of them did in the nature reserve.[98]

8.6.2.2 Studies with Leptodactylus latrans

Trypomastigotes of *Trypanosoma* sp. were observed (Protozoa: Kinetoplastida: Trypanosomatidae) in blood smears of adult specimens of *L. latrans* in agroecosystems from Argentina.[99] Trombiculid mites of *Hannemania* sp. have been reported in adults of *L. latrans* from the argentine provinces of Buenos Aires, Entre Ríos, Jujuy, and Santiago del Estero.[100–102] With regard to fungus infection, the presence of the chytrid fungus has been reported in *L. latrans* in Argentina.[103] It was the first report of chytridiomycosis in Argentina and represents the southernmost record of this fungus for South American amphibians. Amphibian antimicrobial peptides have been isolated on the skin secretions of *L. latrans* adults. The skin secretions of *L. latrans* contain ocellatins, which inhibits the growth of reference strains of bacteria (*Escherichia coli* and *Staphylococcus aureus*).[104–106]

Lajmanovich *et al.*[107] detected residues of chlordane and endosulfan in fatty tissues of five wild *L. latrans* in Entre Ríos Province, Argentina. Correia *et al.*[108] studied the possibility of using tissues of *L. latrans* as indicators of metal pollution in the central south of Bahia, Brazil. They determined the concentrations of manganese, chromium, zinc, nickel, copper and iron in

tissues (skin, muscles and viscera) and concluded that the viscera represent a good alternative for use in biomonitoring surveys.

8.6.2.3 *Studies with* Hypsiboas pulchellus

Brodeur *et al.*[109] evaluated the impacts of the herbicide glyphosate and subsequent intense drought on *H. pulchellus* inhabiting an agricultural landscape. They examined a series of organismic indices (stomach content index, hepatosomatic index, body fat index, gonadosomatic index, condition factor) as well as biomarkers of oxidative stress (hepatic catalase activity and reduced glutathione [GSH] content), exposure to contaminants (hepatic glutathione-*S*-transferase activity), and genotoxicity (micronuclei frequency). No significant differences were observed in the parameters measured when comparing frogs sampled before, and 2 and 15 days after glyphosate application. However, anurans sampled in the same site 2 months later, when a drought was at its peak, presented a decrease in stomach content and hepatosomatic index, as well as an increase in hepatic catalase activity, hepatic GSH content and micronuclei frequency in peripheral circulating erythrocytes. These findings clearly demonstrated that drought is challenging to these anurans.

In Uruguay, Borteiro and coworkers[110] presented evidence of coinfection of *H. pulchellus* by fungal-like parasites of the order Dermocystida (*Amphibiocystidium* sp.) and the fungus *Batrachochytrium dendrobatidis*. This report was the first of dermocystids in Neotropical amphibians since 1940, the first evidence of the fungus for central and northeastern Uruguay, and the first coinfection of *B. dendrobatidis* with other eukaryotic skin pathogens in free-living amphibians.[110] In another study, Borteiro *et al.*[111] reported infection by *Ichthyophonus* sp. in *H. pulchellus* in southern Uruguay. They evidenced a large subcutaneous mass over the urostyle and dorsal musculature comprised of parasitic cysts with mild granulomatous inflammation. There have been no prior reports of *Ichthyophonus* in South America, so the impact of these pathogens on individuals and populations of native amphibians is unknown.[111]

López *et al.*[112] studied amphibian trophic ecology in a range of human-altered wetlands. They analyzed changes in resource availability and use and the population abundance of *H. pulchellus* amongst others anurans, from six wetlands that differed in type and degree of human disturbance. Diet composition of *H. pulchellus* showed significant variation among sites and was correlated with resource availability. These results suggest that species such as *H. pulchellus* which are able to adjust their diets according to prey availability may present an adaptive advantage in changing environments associated with anthropogenic disturbances, namely urbanization, agriculture, and livestock grazing.[112]

8.6.2.4 *Studies with* Rhinella fernandezae *or* Rhinella dorbignyi

Cabagna Zenklusen *et al.*[99] observed microfilariae (Nematoda Filarioidea) in adults of *R. fernandezae* from sites where agrochemicals had been used for a

long time. Additionally, hematology and blood cell cytochemistry have been described for *R. fernandezae* inhabiting natural reserves from the Espinal and Delta Islands of the Paraná River, Argentina.[113]

Sánchez *et al.*[114] demonstrated that *R. fernandezae* from agroecosystems and natural wetland sites adjacent to monoculture zones presented lower testicular volume, a lower number of poorly developed seminiferous tubules, poorly developed primary spermatogonia and spermatids, and fewer primary spermatocytes compared to specimens from a natural forest. These anomalies of gonadal form and function in *R. fernandezae* might affect reproductive success.

Regarding the trophic dynamics of *R. fernandezae*, Peltzer *et al.*[115] compared feeding habits between a soybean field and a native forest in Santa Fe Province, Argentina. Lepidopteran larvae were the predominant item in the soybean field, whereas collembola, isopods and snails prevailed in the diets from forests. This small toad maintained a preference for a few prey types, acting as a trophic specialist in soybean.

8.6.2.5 *Studies with* Rhinella arenarum

The mite *Brasiliensis desantisi* has been reported in adults of *R. arenarum*, from Buenos Aires, Argentina.[101] In another study, Bionda *et al.*[116] analyzed the presence, types and frequencies of abnormalities in an urban population of *R. arenarum* in Villa Dalcar Lake from central Argentina. The lake received urban waste and agricultural runoff, and water analysis revealed high levels of phosphorus. They registered a high rate of ectromelia, ectrodactyly, polyphalangy and amelia, of which ectromelia and ectrodactyly were most commonly observed abnormalities.

The inhibitory effects of copper, cadmium and zinc on metabolism through the pentose phosphate pathway were evaluated in the ovaries of adult *R. arenarum* females. Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase enzymes have been proposed as biomarkers of effect and exposure for Cu and Zn toxicity.[117,118] This enzyme is inhibited by long-term exposure of females to zinc in Ringer solution. As the result of this inhibition, the oocytes are subjected to oxidative stress and respond with an increase of 50% in GSH content.[117–119] In addition, some other metabolic biomarkers of metal exposure respond to sublethal concentrations. δALAD activity is decreased, and the free erythrocyte protoporphyrin (FEP) level is increased by sublethal exposure of *R. arenarum* adults to lead.[120]

Cabagna *et al.*[121] compared hematological parameters and plasma cholinesterase activity in adult *R. arenarum* from control and agricultural sites. Mean plasma cholinesterase activity did not vary amongst toads but blood parameters (hematocrit, hemoglobin concentration, white blood cells, and heterophils) from agricultural sites differed from the control site. In a recent study, Salinas *et al.*[122] also used hematologic parameters to compare the health status of *R. arenarum* from different localities of Córdoba Province, Argentina. They demonstrated the existence of differences in leucocytes types and counts amongst sites differently impacted by anthropogenic activities.

Two different studies[123,124] employed the micronucleus test and erythrocytic nuclear abnormalities to compare the health of populations of *R. arenarum* from sites with different degrees of environmental alteration. Results showed that an association between frequencies of micronucleus and erythrocytic nuclear abnormalities and the degree of environmental alteration recorded for the sites studied.

Bionda *et al.*[125] analyzed population demography of *R. arenarum* associated with agricultural systems in Córdoba Province, Argentina. Population projections were unfavorable for soybeans sites. In another similar study, Bionda *et al.*[126] determined age structure, growth and longevity in the common toad, *R. arenarum*, from a suburban pond located in the Pampa plains, central Argentina during two breeding seasons. They found that males and females showed different morphological and life history traits, such as age at maturity and growth rates, depending on the year.

8.6.2.6 Studies with More than One Model Species

Lajmanovich *et al.*[127] determined the normal levels of butyrylcholinesterase, carboxylesterase, and glutathione *S*-transferases activities in three South American toad species, namely *R. arenarum*, *R. fernandezae* and *R. schneideri*, in order to establish reference values for field pesticide monitoring purposes.

The types and prevalence of abnormalities in amphibian populations from agricultural and reference areas were examined by Peltzer *et al.*[128] They recorded 16 types of abnormalities in 15 anuran species, including *H. pulchellus*, *L. latinasus*, *L. latrans*, *R. arenarum*, and *R. fernandezae*. Ectromelia was the most common abnormality found. Agostini *et al.*,[129] for their part, detected nine types of abnormalities, of which the most frequent were those occurring in limbs. In species such as *H. pulchellus*, *L. latrans* and *R. fernandezae*, they demonstrated a prevalence of abnormalities that was significantly higher in cultivated than in reference areas. Such an increased prevalence of abnormalities was, however, not detected by Brodeur *et al.*,[102] who examined *H. pulchellus*, *L. latinasus*, *L. latrans*, and *R. fernandezae* from another set of cultivated fields. The study by Brodeur *et al.* instead established the presence of a reduced body condition in frogs from agricultural lands. This reduction of body condition was accompanied by an alteration of enzymatic biomarkers in *H. pulchellus* and *L. latrans*, suggestive of an impact of pesticide contamination on the frogs.[102]

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CHAPTER 9

Odontophrynus cordobae *(Anura, Cycloramphidae): A Suitable Model for Genotoxicity in Environmental Monitoring Studies*

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9.1 Biomarkers as a Tool to Assess the Impact of Environmental Contamination

With the advent of the Industrial Revolution and the development of new production methods, environmental impact of anthropic origin has dramatically increased, as well the risks associated with it.[1] The intensification of environmental pollution is not only attributed to the advances in agricultural and industrial technologies, it is also the result of changes in human living conditions and lifestyle. Progressively, human contamination has diversified into different forms creating the immediate necessity to

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establish an effective evaluation approach to determine diagnostic parameters that consider the effect of human activities that affect the health of ecosystems and populations.[2,3]

Therefore, environmental pollution has become one of the major concerns of modern society, particularly, the exposure to genotoxic agents.[4–6] Genotoxic agents are chemical, physical or biological agents that cause direct or indirect damage to genetic material in sublethal doses.[1,7,8] Consequently, genotoxicity is the ability of these agents to interact with the DNA causing structural and functional modifications in both germ and somatic cells. The modification of the hereditary material of germ cells (eggs, sperm), and the cells that originate them, may increase the occurrence of genetic, monogenic, chromosomic, and multifactorial diseases in future generations. Moreover, there is a strong association between DNA modification in somatic cells, cancer and chronic degenerative diseases.[9]

Chromosomic damage owing to inefficient or inappropriate repair mechanisms is manifested during cellular division and represents a measure of the accumulated genotoxic effect.[10] Alterations in the number or the structure of chromosomes are revealed at the cellular level, as cytologically recognizable macrolesions (chromosome aberrations, sister chromatid exchanges, micronuclei), which can be detected through cytogenetic studies.[11] Thus, if the damage is not repaired or persists, it is possible to perform bioassays (biological systems assays) to demonstrate the cellular response to the genotoxic effect.

The need to investigate and detect the impact of chemical contamination in the natural environment has led to the development of markers or indicators to measure the biological effects of contaminants in living organisms (biomarkers). Conventional methods of environmental monitoring, such as measurement of chemical parameters, evaluate contaminant levels and environment quality but they cannot determine the correlation between them. Biomarkers, on the other hand, can be used to determine this correlation between cause and effect.[12,13]

The concept of biomarkers is based on the fact that toxic contaminants produce peculiar responses in the cells and organisms that are exposed; these responses can be detected and measured through bioassays, which provides evidence about the effect and the exposure; it also allows evaluation of the potential risk of diverse environmental exposures.[11,14] According to Hartl, biomarkers are powerful tools to detect the impact of the exposure to a particular substance or to a mixture of them at sublethal doses, making it possible to evaluate the seemingly minimal effects in an organism (sublethal or subtoxic).[15]

In recent years, new biomarkers of genotoxic effect have been defined; these are employed as tools to detect damage caused by exposure to genotoxic agents through variation in cellular responses. These biomarkers can show alterations in DNA that range from point mutations to changes in the genetic diversity of a population, and they are widely applied in genetic toxicology as well as in genetic ecotoxicology.[4,16,17] Regarding ecotoxicological

biomarkers, they have been studied in species that are ecologically relevant through different bioassays to detect and identify genotoxic substances in water, soil and air, showing early cellular manifestations of genotoxicity.[1,16,18–22]

9.2 Amphibians are Suitable Organisms to Evaluate the Genotoxic Effects of Environmental Contaminants

Selecting the appropriate organisms to bioassay is one of the main problems in the biomonitoring of genotoxic contaminants. Variations in species sensibility and differences in metabolic rate, physiological conditions or target organs may generate unreliable results.[23] The organisms that are subject to genotoxicity assays must be chosen by selection criteria that allow us to asses, in a reliable way, the genotoxic capacity of a tested substance based on their response.[24] For this purpose, several cytotoxicity and genotoxicity biomarkers have been employed in organisms like fish and amphibians.[25]

Amphibians, in particular, are among the most sensitive species to environmental changes, mainly owing to an early stage of development that occurs in the water and also to their highly permeable skin. In fact, environmental contaminants have been pointed out as the most important cause of the decline of amphibian populations.[26–28]

The complex life cycle of most amphibians, particularly frogs, allows the larval and adult stages to occupy entirely different ecological settings, providing a unique situation among tetrapods.[29] During the larval period, anurans exhibit a series of dramatic morphological changes.[30] The continuously changing appearance of embryos and larvae during ontogenesis necessitates a method to quantify the progress of that development.[31] Staging is the recognition of certain morphological landmarks that appear useful to compare the sequence of events in a developmental continuum.[29] Research on the anatomy and development of the larvae can provide valuable insights into systematic and functional diversification in closely related anurans' taxa.[32] These types of studies are essential to many researches involved with frog life-history materials and teratogenicity tests in toxicological studies.

Amphibians are more closely related to water and wetlands than most reptiles, birds and mammals. The thin skin in adults, as a highly permeable organ involved in hydric balance and breathing, added to a lifestyle between aquatic and terrestrial environments, a restricted ''home ranch'' and the limited ability to spread out in the environment, make amphibians a suitable organism for environmental biomonitoring.[33–35]

Even though environmental contamination interferes with the normal growth and development of amphibians and with their susceptibility to diseases, genetic damage induced by chronic exposure to agrochemicals is probably the most important biologic effect that contributes to the decrease in the number of amphibian populations.[25]

The blood of amphibians includes erythrocytes, leukocytes, thrombocytes and heterophils, white acidophilic granules that can be considered analogous to neutrophils in mammals. Both erythrocytes and thrombocytes are nucleated cells. Hematopoiesis in amphibians has some similarities to those of birds and mammals. The kidney is the main organ of blood production during the larval stage in amphibian species. In adults, the production of erythrocytes and thrombocytes takes place mainly in the spleen while the liver is a secondary organ for this activity. Hematopoiesis in the bone marrow is an evolutionary novelty in amphibians, and typically occurs after metamorphosis or post-hibernation. Anuran species have nucleated, ellipsoidal, oblate and biconvex erythrocytes, with a size of approximately 15×22 μm and usually mature in peripheral blood. Their life time is longer than that in birds and mammals, ranging from 700 to 1400 days. During hibernation there is a deceleration of the hematopoiesis and an increase of erythrocytes lifetime in peripheral blood as a consequence to the reduction of metabolic activity during this period.[36,37]

9.3 Relevant Features of *Odontophrynus cordobae* for Genotoxicity Studies in Environmental Monitoring

The genus *Odontophrynus* is endemic to southern and eastern South America and consists of 11 recognized species so far. *Odontophrynus* species are clustered in three groups based on external morphology: americanus, cultripes, and occidentalis. The americanus group is represented by four species: *Odontophrynus americanus*, *O. lavillai*, *O. cordobae* and *O. maisuma*.[38] The recently described *O. cordobae* is cryptic with its polyploid counterpart, *O. americanus*, and it is found in central and northwestern Córdoba and southern Santiago del Estero provinces.[38–46]

Odontophrynus americanus includes diploid and tetraploid forms. Diploid populations of central Argentina were revised by Martino and Sinsch in 2002 and described as a new species, *O. cordobae* (Figure 9.1). The distribution of *O. cordobae* is restricted to central and north-western Córdoba and some populations in the southern Santiago del Estero province, Argentina.[42] *Odontophrynus cordobae* is morphologically cryptic with its polyploidy counterpart *O. americanus*, which is widely distributed in Argentina.[43] Some of the southernmost populations of *O. cordobae* coexist with populations of *O. americanus* in central-western Cordoba.[47–49]

Martino and Sinsch reported that *O. cordobae* breeds exclusively in small streams, and often in syntopy with *O. americanus*.[42] Breeding sites were found in backwaters along streams and in temporary and permanent ponds of varying size. Most of these sites had riparian and semi-submerged vegetation that provided protection to callers. Males and females approached breeding sites at the same time, preferentially moving through the waterway.

Figure 9.1 Photograph of *Odontophrynus cordobae*.

Amplexus started as soon as acoustic activity began. Eggs were deposited individually and sank to the bottom of streams and temporary ponds.[47,49]

Odontophrynus cordobae combines the proper biologic and ecologic features to conduct laboratory and field studies (*in situ*).[26,27,50] These animals are abundant and easy to collect, simple to manipulate under laboratory conditions and they present few difficulties for blood extraction using minimally invasive techniques.[51]

In environmental monitoring studies, it is recommended to consider the karyotype for the correct selection of the species tested in the bioassay. According to Udroiu, in those species with a high number of small chromosomes it can be difficult to observe if structural and numerical alterations occur; because of this, the species with less and larger chromosomes are recommended.[16] Thus, it is correct to infer that karyotype features must be evaluated in those species used for this assay.

O. cordobae is a diploid species ($2n = 22$). Pairs 1, 5–7 and 10–11 show metacentric morphology and pairs 2–4 and 8–9 are submetacentric. Chromosomes 1–4 are large (relative chromosomic length from 14.09 to 10.64%), pairs 5–7 are medium length (relative chromosomic length from 8.70 to 7.12%), and pairs 8–10 are the smaller ones (chromosomic length from 5.15 to 3.53%). The chromosomes' meiotic behavior through prophase I in *O. cordobae* shows the presence of 11 bivalents and in secondary spermatocytes 11 dyads were counted. Secondary constrictions were observed in chromosomes of pair 4.[52,53]

In environmental biomonitoring studies that use amphibians as bioindicators, two genotoxic biomarkers have been traditionally used: alkaline electrophoresis of individual cells (comet assay) and micronucleus test.[3,54–56] Micronucleus test stands as one of the favorite and most frequently used techniques to detect the presence and frequency of micronuclei

produced by clastogenic and aneugenic agents on cells in the interphase, as well as nuclear abnormalities. It is a reliable, uncomplicated, rapid and sensitive tool that is based in simple morphologic standards.[1,16,23,57–70] This assay was selected as the gold standard test in mutagenesis by the International Workshop on Genotoxicity Test Procedures.[71]

Micronuclei (MN) are considered as a genotoxic effect biomarker at the subcellular level. This biomarker can be measured with cytogenetics techniques and the increase in the frequency of micronucleated cells is considered an early response to chromosomic damage.[8,72] MN represent the only biomarker that detects the consequences of clastogenic and aneugenic effects during interphase.[60,62,63]

This assay was originally described by Boller and Schmid[73] and Heddle[74] to detect the genotoxic potential after *in vivo* exposure, studying bone marrow erythrocytes from mammals, nowadays, is widely used to evaluate the potential genotoxicity of chemical agents in several organisms groups.[23,71,75,76] The micronucleus test was proposed to be applied on amphibians for the first time by Jaylet *et al.*,[77] who tested erythrocytes of *Pleurodeles waltl* larvae in peripheral blood and it has been known as the Jaylet Test ever since.

It has also been tested *in vivo* using peripheral blood erythrocytes as a good indicator of both the genotoxic effect of contaminants in aquatic environments and the genotoxic faculties of chemical substances under laboratory conditions.[26,50,78–81] Specialists like Van Hummelen *et al.*[78] and Zoll-Moreux *et al.*[82] applied the micronucleus test on amphibians to evaluate genotoxic agents in aquatic environments, and today the test is becoming widely used. Genotoxicity assays in amphibians are currently based on *in vivo* MN observation in erythrocytes of peripheral blood.[79]

This biomarker has been employed in amphibian species that inhabit environments that are contaminated periodically with agrochemicals and other chemical substances.[3,37,54,79,81,83] The micronucleus test has been frequently used to detect genotoxicity induced by clastogenic or aneugenic agents in pre-metamorphic anurans and urodele amphibians,[77,79,80,84,85] but was barely tested in post-metamorphic amphibians.[86,87] Peripheral blood collected from amphibians in the post-metamorphic stage is analyzed to determine the micronuclei frequency. The advantage of practicing this assay is that tested animals are released after the sample collection since sacrifice is not required.[88] Furthermore, the interest in studying post-metamorphic specimens lies in the spatial and temporal prevalence of these stages in the agroecosystem, which, added to the particular features previously mentioned, makes *O. cordobae* a suitable species to develop useful biomarkers for environmental biomonitoring.[54]

At the moment, only two studies report the genotoxic effects of chemical substances in Odontophrynus specimens. Cabagna *et al.*[79] evaluated the genotoxic effect of a commercial formulation of cypermethrin in pre-metamorphic larvae of *O. americanus* exposed to four concentrations of cypermethrin. For all treatments, the occurrence of micronuclei was relatively lower (1–3MN per 1000 cells), even for those with a significant increase

in the frequency of micronuclei compared to the negative control group. Bosch *et al.*[54] evaluated the genotoxic effect of a commercial formulation of glyphosate in post-metamorphic individuals of *O. cordobae* and *Rhinella arenarum*. No other studies of genotoxicity in adult forms of these species have been reported. The animals used in this study were those considered young adults of reproductive age, according to their body size. The animals were kept in acclimatization for a period of 15 days; they were placed in 10 L plastic containers lined with damp paper towels to keep the animals hydrated, at ambient temperature and natural photoperiod. During this time, the animals were fed on larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae) bred in the laboratory. Hayashi's recommendations for the treatment of animals were followed.[89] The animals were split into three groups of five individuals each: a negative control group, a positive control group and a treated group. Positive control animals were exposed to 40 mg L^{-1} cyclophosphamide. Treated group individual were exposed to different Roundup® concentrations (100, 200, 400 and 800 mg L^{-1} of active ingredient). Blood samples for the micronucleus assay were obtained from days 2 and 5 of treatment through a small incision in the angularis vein. Finally, the number of micronuclei was determined by analyzing 3000 erythrocytes per animal.

The basal frequency of micronucleated erythrocytes in *O. cordobae* was 0.40 ± 0.18MNE per 1000, higher than that found by Cabagna *et al.*[79] in *O. americanus* larvae. The commercial formulation of glyphosate tested in *O. cordobae* at 200, 400 and 800 ml L^{-1} concentrations was lethal on the second exposure day. The group exposed to cyclophosphamide for 5 days showed significant differences in micronucleated erythrocyte frequency when compared to the control group. *Rhinella arenarum* proved to be more resistant to acute toxicity of the herbicide and there was no mortality in any treatment. However, at a glyphosate concentration of 100 mg L^{-1}, *O. cordobae* had a higher frequency of micronucleated erythrocytes (0.88 ± 0.33MNE per 1000 analyzed erythrocytes) than *R. arenarum* (0.46 ± 0.16MNE per 1000 analyzed erythrocytes). This result is consistent with the response of *O. cordobae* to cyclophosphamide exposure, which showed a significant increase compared to those obtained for *R. arenarum*.[54]

Although both species showed a statistically significant increase in the frequency of micronucleated erythrocytes compared to the basal frequency, this value was higher for *O. cordobae.* Thus, it is suggested that this species has a major sensibility to clastogenic agents in the environment. This result may be owing to an ineffective repair mechanism of genetic damage or to an inefficient mononuclear phagocyte system (responsible for removing anomalous or old erythrocytes from blood circulation) when compared to *R. arenarum*.[90] Evenden *et al.*[91] reported these differences of susceptibility to xenobiotics owing to natural genetic variations, even in intraspecific cases.

The study performed by Bosch *et al.*[54] showed high levels of toxicity and lethality in specimens of *O. cordobae* exposed to Roundup®, which caused

the interruption of the assay and the adjustment on the sampling scheme for *R. arenarum*. Moreover, only minimal signs associated with acute toxicity were observed in *R. arenarum* and there was no lethality at any time; thus, these results prove that *O. cordobae* is much more sensitive than *R. arenarum.*

According to the literature, it is recommended that the studied species show a basal frequency of micronucleated erythrocytes of over 3.5 per 10 000; thus, *O. cordobae* is a suitable organism to be used in the micronucleus assay because the basal frequency of micronucleated erythrocytes exceeded that value.[90]

The use of species in which the spleen does not remove micronucleated erythrocytes from blood is related to the implementation of the micronucleus test in peripheral blood and it has been widely discussed in the literature.[67] Moreover, this ability depends on the spleen's anatomical structure and it may act as a confounding factor while leading to false negatives results.

The OECD[92] established that any mammal species is suitable for micronucleus testing as long their spleen does not remove micronucleated erythrocytes from the blood. According to Udroiu[67] the nonsinusal spleen of amphibians and others aquatic vertebrates does not jeopardizes the micronucleus assay. This fact was confirmed by Bosch *et al.* in their study on *O. cordobae* through comparing the mean frequencies of micronucleated erythrocytes in animals treated with cyclophosphamide and the negative control group.[54]

Another aspect to be considered when the assay is performed in erythrocytes of peripheral blood is the correlation between hematopoiesis variations and the frequency of micronucleated erythrocytes.[67] Since this assay requires mitotic cell populations, alterations in hematopoiesis have a major importance as they may cause confusion. As stated by Udroiu,[67] the implementation of the assay when hematopoiesis is accelerated can lead to false positive results. This could happen, for example, if the blood samples are obtained repeatedly from the same animals at short intervals. In the same way, when hematopoiesis decelerates, the production of micronucleated erythrocytes decreases along with erythrocyte production. Consequently, the implementation of the assay under such conditions may induce false negative results.[36]

9.4 Conclusions

Although conventional methods to evaluate environment quality were based on physicochemical measurements, the current approach is the systematic study of the organisms response evaluated in different organization levels (biomonitoring). The fact that physicochemical schemes cannot detect damage in organisms produced by exposure to xenobiotics is one of the most important premises of biomonitoring. Therefore, it is necessary to

incorporate new methods of evaluating environment quality that complement the traditional ones.[93–95]

The biomonitoring of ecologically relevant species is considered an important tool in the study of environment quality, mainly in some regions affected by contamination of anthropogenic origin. For this reason, ecologists and toxicologist recommend the implementation of biomonitoring, particularly in those environments affected by contamination and that need to be remediated. This approach establishes that selected biological responses, which are measured in different species and in several levels of biological organization, provide information regarding the effects of and exposure to xenobiotics. This information is often related to noxious consequences owing to such exposures. The aim of biomonitoring is to determine the correlation between the presence of a xenobiotic and the response of different biological components in the ecosystems.[95,96]

There are some limitations in the implementation of biomarkers *in situ*, such as modifications in the environment variables, dynamics of populations and communities, biomarker specificity, dose–response relationship or exposure to a mixture of contaminants in environmental conditions.[4,97,98] However, the significance of bioassays to diagnosis and monitoring of environmental quality is based on their ability to evaluate the sublethal effects of contaminants on living organisms.

According to Jha, the development of *in vivo* systems for the diagnosis and monitoring of environmental quality must consider the expression of genotoxic activity in ecologically relevant organisms.[99] These systems consider the real ways of environmental exposure, the effects of metabolism and the efficiency of the DNA repair mechanisms. Despite the great concern regarding the presence of genotoxic agents in the environment, there are few suitable methods that can be used to evaluate genotoxicity in organisms under certain environmental conditions.

The validation of *in vivo* systems, such as the micronucleus test in peripheral blood of *O. cordobae*, provides scientific support for *in situ* studies of the potential risks produced by environmental exposure to genotoxic agents. The statistically significant increase in the frequency of micronucleated erythrocytes in *O. cordobae* exposed to cyclophosphamide in Bosch *et al.*'s study[54] validates the use of post-metamorphic specimens in the micronucleus assay. Moreover, the observation of the basal frequencies of micronucleated erythrocytes in *O. cordobae* will allow the implementation of this biomarker for diagnosis and monitoring of environmental quality. According to the available literature, *O. cordobae* is more sensitive to exposure to genotoxic substances than *R. arenarum*, and the biomonitoring of this species in post-metamorphic stages trough the micronucleus assay can be accomplished without animal sacrifices. Biological and ecological features in the post-metamorphic stages of *O. cordobae* suggest that this is a suitable organism for bioassay in genotoxicity studies, either *in situ* or under laboratory conditions.

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CHAPTER 10

The Direct-developing Frog Eleutherodactylus johnstonei *(Eleutherodactylidae) as a Biological Model for the Study of Toxic, Cytotoxic, and Genotoxic Effects of Agrochemicals*

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10.1 Introduction

Ecotoxicological research using anurans as biological models has mainly focused on the use of larval stages of metamorphic species, which mainly

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inhabit aquatic systems.[1] Agrochemicals are used mainly in terrestrial environments; therefore, it is imperative to identify a terrestrial frog as a laboratory model to enhance our understanding of the potential negative impacts of agrochemicals on the health and sustainability of ecosystems.[2,3] This chapter is aimed at providing information to validate the use of *Eleutherodactylus johnstonei* (Barbour, 1914), commonly named The Antillean Coqui, as an innovative model organism in ecotoxicology research and to offer standard guidelines for housing and rearing this species under laboratory conditions.

10.2 Natural History of the Antillean Coqui

Eleutherodactylus johnstonei (Figure 10.1) is a frog member of the family Eleutherodactylidae in the monophyletic clade Brachycephaloidea, commonly

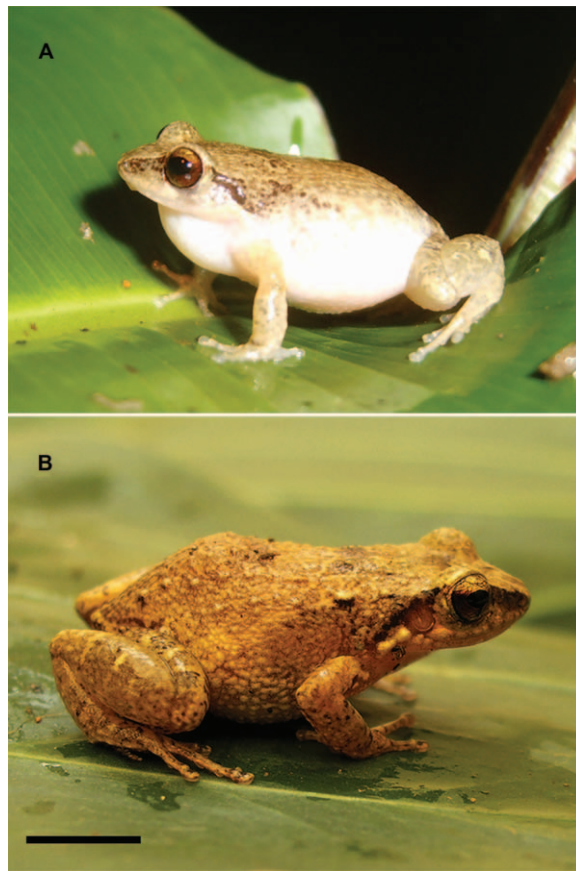

Figure 10.1 Adult male (A) and female (B) of *E. johnstonei* from ''Parque de las Orquídeas,'' Bucaramanga, Colombia. Scale bar = 1 cm. (B) courtesy of Laura Valencia.

named terraranas.[4–6] With more than 1000 species, this group comprises around one third of all New World frog species, and therefore exemplifies one of the taxonomic groups best represented in amphibian communities through its distributional range.[5–7] Species within this clade have terrestrial breeding and direct development (except for *Eleutherodactylus jasperi*, which is a viviparous species), avoiding the tadpole stage.[5–8] Direct development in this taxon is a derived feature that presumably evolved once during its life history.[8] Native populations of this species inhabit mostly disturbed areas but also occur in forested areas, usually in gaps and clearings, while introduced populations tend to inhabit mainly altered habitats, often associated with house gardens and parks in urban areas or residential places.[9–12] This species has a carnivorous generalist diet, which probably depends on prey availability in the environment. The most common prey are Formicids, Homopterans, Orthopterans, Dipterans, Hemipterans, Dermapterans, Collembolans, and other Arthropods.[12–14]

The reproductive activity of this species is flexible, having populations with continuous reproductive cycles in Colombia[11] and with seasonal reproductive cycles in Barbados.[15] Mature males attract gravid females through advertisement calls comprising two contiguous notes often follows by short click calls.[16–18] Males defend calling territories against conspecifics through vocal and visual displays, as well as by physical fighting.[17–20] Courtship starts when gravid females come near and make physical contact with a calling male, then the male stops its advertisement calls and initiates softer calling to lead his mate into a potential oviposition site where the axillary amplexus occurs.[20,21] Oviposition sites included leaf litter, orchid root masses, hanging ferns rhizomes, bromeliad axils, and small natural and artificial cavities (*e.g.*, under decaying logs, coconut husks, boards, rocks[20]). Eggs are externally fertilized and the embryo hatches as a fully formed miniature froglet.[20,21] Egg attendance is uniparental but provided by both males (in a major intensity) and females, beginning at opposition and persisting until hatching, or even between 1–8 days beyond.[21]

10.3 Geographic Distribution

The geographic distribution of *E. johnstonei* has experienced changes over time, mainly for two reasons. First, many populations of this species were for over 30 years erroneously treated as *Eleutherodactylus martinicensis*.[22] Second, this species has been expanding its distributional range continuously during the last few centuries.[22–25] This species is native to the Lesser Antilles, but their exact origin remains unclear because in this region it is difficult differentiate native and introduced populations.[22–25] Presumed native populations are known to occur in several island of the Lesser Antilles (Figure 10.2), including Saint-Martin, Sint Maarten, Saba, Sint Eustatius, Saint Kitts, Nevis, Antigua, Montserrat, Martinique, Saint Lucia, and Saint Vincent and the Grenadines.[22] Invasive populations are thought to occur in numerous Antillean islands (Bermuda, Jamaica, Anguilla, Guadeloupe,

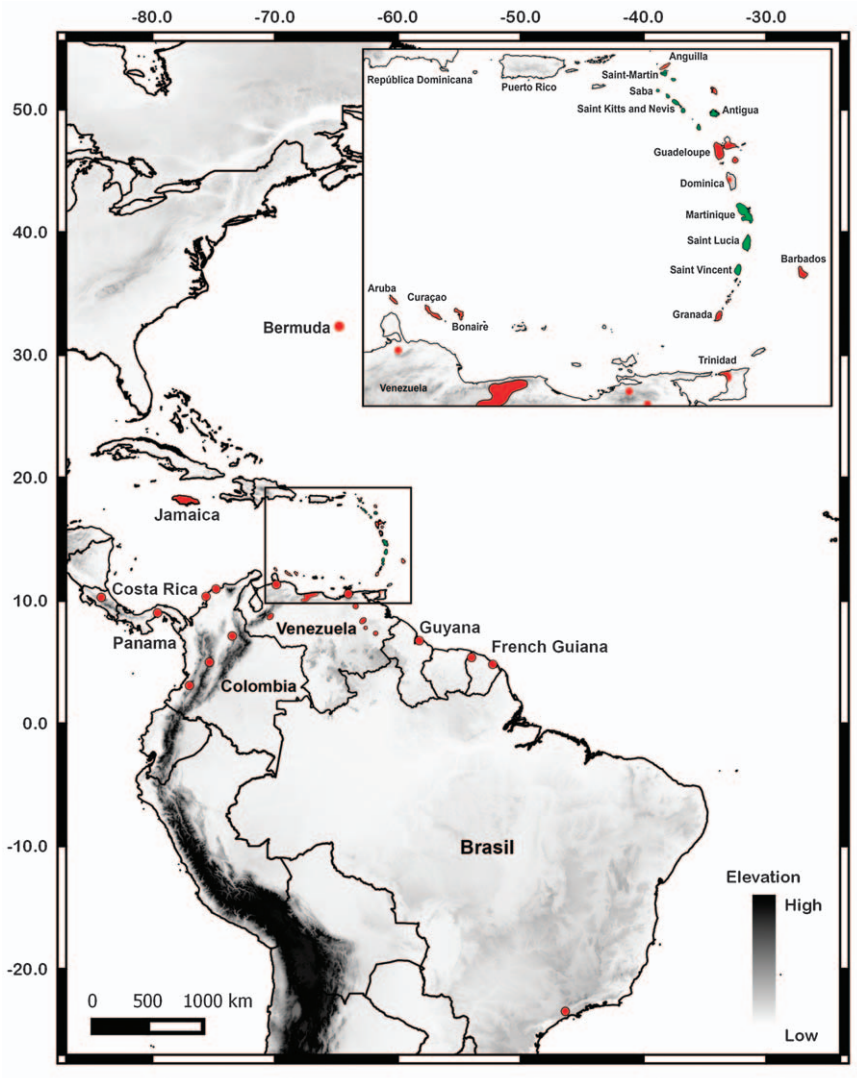

Figure 10.2 Map showing the geographic range of *E. johnstonei* in South America. The green polygons indicate the presumed native range of the species.[22] The red polygons represent introduced populations (see text). The native geographic range is shown as the boxed area in the inset map on the upper right.

Dominica, Barbados, Granada, Trinidad and Tobago, Bonaire, Curaçao, and Aruba) and into adjacent mainland Central (Costa Rica and Panama) and South America (Colombia, Venezuela, Guyana, French Guiana, and Brazil) (Figure 10.2); where it is known to occur from sea level up to 1750 m elevation in the Venezuelan Andes.[22–32]

10.4 The Antillean Coqui as an Invasive Species

Eleutherodactylus johnstonei has been considered an introduced species since the time of its original description in early 20th century. The first documented introduction of the species occurred around 1885, when the frogs were introduced in Grenada.[33] From this point forward, the species was introduced into a variety of Caribbean Island and adjacent Central and South America mainland, apparently by unintentional (*e.g.*, ornamental plant trade, roadside litter transport, nursery trade, increased goods exchange) and intentional human activities (*e.g.*, illegal frog trade, biocontrol agent), rather than active dispersal.[22,25,28,34] As a consequence, the species is currently known as the most widely distributed frog in the eastern Caribbean.[24] The high colonizing success of the species appears to be related to its flexibility in reproductive activity, generalist diet and microhabitat use, tolerance of xeric conditions, direct developing eggs, and parental care of the clutch, allowing it to establish once introduced.[10–12,24,28]

Established invasive populations of the species are often confined to urban areas,[10–12,24,30,34] but also may occur in rural and natural areas.[14,35] Studies have shown that whereas in some introduced localities the species has not spread significantly during the last decade (*e.g.*, French Guiana[34]), in others the species appears to spread (*e.g.*, Jamaica[22] and Trinidad[30]). Actually, based on recent climate models, the species is expected to significantly extend its distributional range into the Andes of Colombia, Ecuador and Venezuela, which are the most suitable areas in terms of climate and habitat modification.[24] The low temperature of the Andean mountains in Colombia and Venezuela has been suggested as a potential barrier to the frog's active dispersal.[10,24] However, this barrier can be easily broken by human-mediated introduction, which remains as a main concern because it may promote further invasions.[24]

10.5 Conservation Status and Concerns

Eleutherodactylus johnstonei is classified as Least Concern (LC) according to the International Union for Conservation of Nature (IUCN) Red List.[25] This assessment was based on the fact that the species ''is common and adaptable with presumed large populations, and it is unlikely to be declining to qualify for listing in a more threatened category''. Several studies have showed that the species do not face significant threats;[22,25,30] instead this frog may actually benefit from the human footprint.[24] Continued degradation of natural habitats represents the main concern because it promotes the expansion of the species once habitats are modified, leading to competitive interactions with the native species that still remain in the newly degraded areas.[22,26,34,36] Actually, some studies have suggested that in recent modified habitats the species may displace native frogs (*e.g.*, *Pristimantis euphronides* and *P. shrevei*) through competitive interactions.[22,26,36] This species is known to carry the chytrid fungus *Batrachochytrium dendrobatidis*,

but they apparently are not severely affected by chytridiomycosis, acting as a reservoir for the pathogen.[37]

10.6 The Antillean Coqui as a Model in Ecotoxicology

Selecting an appropriate model organism for ecotoxicological research is challenging. This selection should integrate a series of key factors (*e.g.*, study scope and organism sensibility, accessibility, and ecological relevance) aimed to guarantee its applicability and usefulness.[38–41] Among amphibians, there are no standard experimental model organisms as strongly established as *Xenopus leavis*. This species complex[42] has received considerable attention in ecotoxicology owing to the nearly ideal qualities for a model organism in genetics, developmental biology, medicine, and ecotoxicology.[43] Unfortunately, in ecotoxicology research there is no such thing as a perfect model and *X. laevis* is not the exception. This species (as others in the family Pipidae) has a derived morphology and biology associated with a fully aquatic lifestyle.[42–45] Furthermore, pipid frogs account for only 0.5 percent of the global amphibian diversity[6] and as a consequence do not represent the vast majority of amphibian communities nor its ecological requirements.

Eleutherodactylus johnstonei exhibits some characteristics expected from a useful model organism (Table 10.1). Several ecological and behavioral traits of this species are well-known, which facilitates its maintenance and breeding under laboratory conditions without major technical requirements and maintenance costs.[2,3,46] Under optimal conditions (see below), the species is capable of ovulating throughout the year and a mass of eggs (41 ± 12 SD [standard deviation] eggs) can be obtained twice a month (Meza-Joya, unpubl. data), from which embryos and neonates are easily raised.[3,46] Their eggs are large-sized (3.6 ± 0.5 SD mm of diameter), which facilitates their observation and probably micromanipulation. During early development, eggs are nearly unpigmented and many features (*e.g.*, cardiovascular system, eyes, limbs, and endolymphatic calcium deposits), are clearly visible

Table 10.1 Comparison of selected features of *E. johnstonei* with those from the well-established amphibian model *Xenopus laevis*. References are given in the text.

Feature	*X. laevis*	*E. johnstonei*
Clutch frequency	3 months	0.5 months
Clutch size	>1000 eggs	41 ± 12 eggs
Egg size	1.3 mm	3.6 ± 0.5 mm
Embryonic developmental time	3 days	15 ± 0.9 days
Larval developmental time	55 days	—
Taxonomic representativeness	Low	High
Keeping cost	Medium	Low
Access to specimens	High	Medium
Genetic background	Well known	Poorly known
Life style	Aquatic	Terrestrial
Reproductive mode	Biphasic development	Direct development

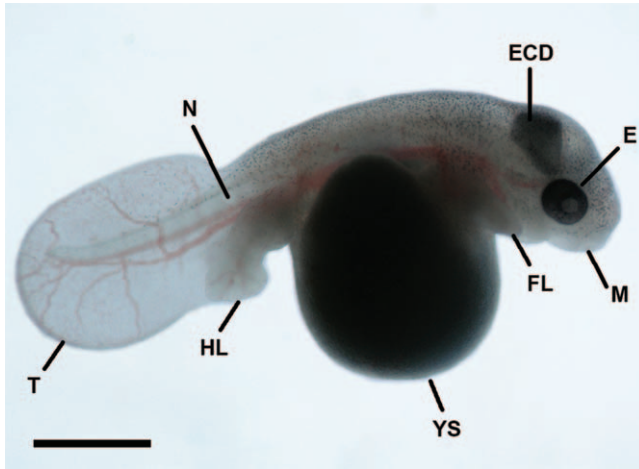

Figure 10.3 Lateral view of Townsend and Stewart's[51] Stage 8 embryo of *E. johnstonei* showing some external and internal features: endolymphatic calcium deposits (ECD), eyes (E), fore-limbs (FL), hind-limbs (HL), mouth (M), notochord (N), tail (T), and yolk sac (YS). Note cardiovascular system irrigating the whole embryo's body. Scale bar = 1 mm.

(Figure 10.3). This fact, coupled to the fast embryonic development of this species (15 ± 0.9 SD days), allows for rapid teratogenic analyses.

This species also shows high sensitivity to reference mutagens and herbicides.[2,3] Several lethal and sublethal end-points has been evaluated,[2,3] and many other potential end-points await assessment (see below). This species represents a generalized model for other terraranas, one of the most diverse and threatened vertebrate groups in the world.[5,6,47] Furthermore, this species displays one of the most representative life history modes in amphibians (*i.e.*, terrestrial direct-development), from which extrapolations can be made to other direct-developing frogs. Owing to their terrestrial development, eggs and embryos are more susceptible to direct exposure to agrochemicals. Finally, but no less importantly, this invasive species does not actually face significant threats,[25] which enables its use as research model.

Beyond these strengths, there are some general limitations with using *E. johnstonei* as a model organism. Today, this species is not accessible to researchers outside its distributional range. Thus, at least for the present time, it is suitable as a model organism only in some Neotropical regions, but the easy breeding of this species under laboratory conditions may facilitate its future raising and commercialization for research around the world. Their reduced clutch size (see above) makes teratogenic studies more challenging. To overcome such a problem, it is possible to establish several breeding colonies at a time to ensure enough organisms for research. Finally, there is so far no information about the genetic background of this species (*e.g.*, genome structure and size, population genetic variation and structure), which has been identified as a key factor underlying the

susceptibility and resistance thresholds to pollutants.[48] Further genetic studies undoubtedly can help to overcome this limitation.

10.7 Collection, Maintenance, and Reproduction in Captivity

10.7.1 Collection and Sex Determination

The collection methods for *E. johnstonei* described in this section have been successfully used by the members of our laboratory during their research.[2,11,12,46] These methods are similar to those from other studies in Barbados, Guyana, and French Guiana,[15,20,21,34] thus probably can be applicable to any area where the species is found. Previous to collecting specimens, it is important to contact the corresponding environmental authority because this activity requires scientific collecting permits in several countries. Adult specimens are mainly collected during their nocturnal activity period, using headlamps. Daytime collection is time-consuming and less efficient in term of adult frogs, but often results in the encounter of developing clutches. We recommend implementing opportunistic visual and acoustic encounter surveys, searching actively for the frogs in all potential microhabitats. Males calling are easily found in exposed mid-elevate perches, whereas silent females are more difficult to locate, but are often placed in the vicinity of vocalizing males at lower perch heights. Once located, frogs are easily caught by hand, but scoop nets can be helpful if the researcher has no experience catching anurans. Specimen sex can be easily determined in the field based on sexually dimorphic traits (*e.g.*, females larger than males, vocal sac present in males) and by listening for the male's calls.[2,3,46]

10.7.2 Taxonomic Identification

For practical proposes, we provide a brief description of the external morphology of this species, but strongly encourage those researchers without taxonomic expertise who are interested in the use of this frog as a laboratory model to contact a local herpetologist for the proper identification of the collected specimens. *E. johnstonei* is a medium-sized frog with an adult snout-vent length (SVL) of 17–29 mm for males and 23–32 mm for females.[11] The generalized coloration patterns in life are as follows: dorsum brown to grayish-brown; scapular chevron, often in combination with a second chevron; pale median hairline or prominent pale dorsolateral stripes; iris gold with black reticulations.[9,49] Diagnosable morphologic characters of the species are:[9,49] medium-sized hind limbs (tibia mean length 44.3 ± 3.4 SD percent of SVL); head relatively wide (head mean width 38.9 ± 2.0 SD percent of SVL); inguinal glands absent; dorsum smooth to slightly tuberculate; snout truncate in dorsal view; eyelids with abundant small rounded tubercles; tympanum distinct; finger and toe disks small and rounded; digital webbing absent; abundant small plantar tubercles; conical outer metatarsal tubercle

shorter than elongated inner metatarsal tubercle; tarsal fold absent; adult males with vocal slits, subgular vocal sac, and nuptial pads absent. For a detailed description of the species, consult Schwartz[9] and Savage.[49]

10.7.3 Maintenance and Reproduction in Captivity

The maintenance methods described in this section were implemented at Laboratorio de Microbiología y Mutagénesis Ambiental of Universidad Industrial de Santander (Bucaramanga, Colombia) as a part of the studies of Valencia *et al.*[2] and Meza-Joya *et al.*[3,46] Frogs can be successfully kept and raised at room environmental conditions of 12 h light/dark photoperiod and 24 ± 2 SD °C. Field-collected frogs are maintained in glass terrariums ($50 \times 40 \times 30$ cm, 0.06 m^3 or 16 gallons) with holes at the bottom to allow drainage (Figure 10.4A). Humidity within terraria can be maintained covering their bottom with a first layer of gravel (near 3 cm) and a second layer of commercial sterile humus (near 5 cm), as indicated by Elinson *et al.*[50] A third layer of leaf litter (near 5 cm) can be supplemented to serve as a water reservoir. Each terrarium is supplemented with bromeliads (one or two) and

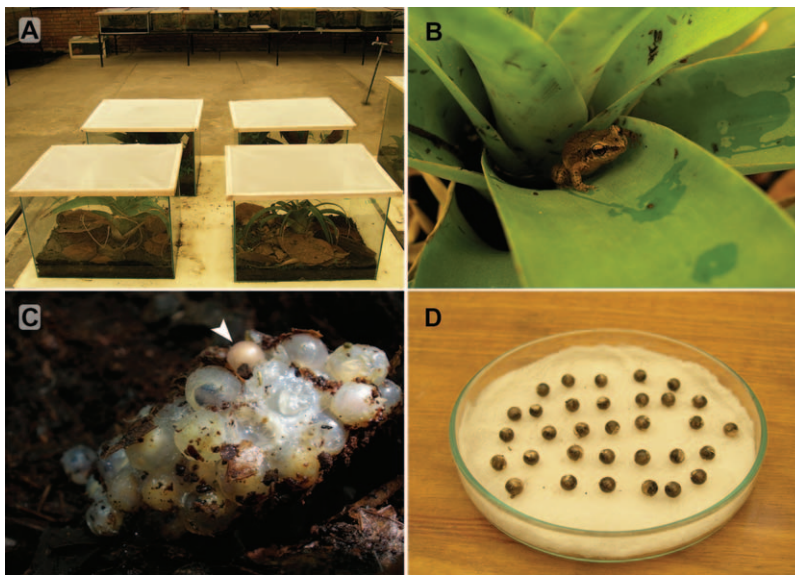

Figure 10.4 Overview of the captive breeding facility for *E. johnstonei* at Laboratorio de Microbiología y Mutagénesis Ambiental of Universidad Industrial de Santander, Bucaramanga, Colombia. Glass terraria housing breeding colonies of frogs set up with substrates, leaf litter, and bromeliads (A). Adult female using the supplemented bromeliads as refuge (B). Egg mass obtained from captive breeding pairs. The arrow indicate an unfertilized egg (C). Individualized developing eggs transferred to a Petri dish with a tiny layer of cotton covering with sterile gauze soaked with distillated water (D).
(A) and (B) courtesy of Laura Valencia.

small fragments of PVC pipe and tiles to act as refugia and oviposition sites (Figure 10.4B). Terraria are covered with mesh screens blocked with metallic bars to allow ventilation and prevent escapes, respectively. We recommended placing the terraria on metallic frames of near 40 cm height to prevent infestation of insects.

The number of frogs in each terrarium can vary depending on the research objective. For breeding purposes, a stock consisting of three females and two males can be kept per terrarium, whereas for long-term raising and short-term housing up to 10 specimens can be maintained. Terraria are sprayed with chlorine-free water three times a week simulating rainfall, which apparently induces reproductive behaviors such as male calls. This behavior can be reinforced by simulating the presence of an acoustic competitor *via* conspecific playbacks. Each breeding pair produces a clutch twice a month continuously through the year (Figure 10.4C). It is unknown whether this species responds with ovulation to the administration of human chorionic gonadotropin (hCG). Given the generalist diet of this species,[12] adults, juveniles, and hatchling froglets can be fed *ad libitum* twice a week with living arthropods (*e.g.*, ants, flies, springtails, crickets, spiders, mosquitoes, aphids and cicadas) captured with scoop nets in green spaces.[2,3,46] Under these conditions, hatchling specimens can be successfully raised with survival rates of up to 63% to obtain juvenile and adult specimens (Meza-Joya, unpubl. data).

10.7.4 Handling Embryos

The procedures described in this section include unpublished data from the studies of Meza-Joya *et al.*[3,46] These methods were successfully used to obtain embryos and neonates from clutches with hatchling rates up to 87%. After fertilization occurs, clutches can be transferred from the oviposition site to a Petri dish with a tiny layer of cotton covered with sterile gauze soaked with distilled water. To avoid infestations caused by soil pathogens such as fungi and nematodes, we recommend releasing all the eggs from the clutch with a tiny brush or forceps, discarding non-fertilized eggs (Figure 10.4D). Further contamination can be avoided by immersing the eggs in a 0.1% sodium hypochlorite solution for 5 seconds. Petri dish with individualized eggs are then enclosed in a transparent plastic recipient with small holes in the top to prevent desiccation. Egg recipients can be maintained at room environmental conditions of a 12 h photoperiod cycle and 24 ± 2 SD °C. Developing embryos can be easily staged according to the table of Townsend and Stewart[51] for *E. coqui*, which establishes a total of 15 embryonic stages from oviposition to hatching.

10.8 Applications for Testing Environmental Xenobiotics

Recent studies have highlighted the potential of *E. johnstonei* as a useful organism model for studying both the acute and sublethal response of

terrestrial frogs to environmental xenobiotics.[2,3] Valencia *et al.*[2] developed a modified enzymatic (proteinase K) Comet assay to assess *in vitro* sensitivity of blood cells to standard mutagens. This assay allowed the measurement of DNA strand breakages (DSBs) induced by bleomycin (BLM) and 4-nitroquinoline-1-oxide (4NQO) with high confidence and reproducibility. Meza-Joya *et al.*[3] used this frog as a model organism to assess the toxic, cytotoxic, and genotoxic effects of a glyphosate-based formulation (Roundup®SL–Cosmoflux®411F). This study showed that the tested formulation induce lethal effects (mortality as the median lethal dose, LD_{50}) in adult frogs and neonates. Sublethal effects (*in vitro* and *in vivo*) were also induced at levels lower than the lethal end-point, including cytotoxic (cell mortality as the median hemolytic application dose, HD_{50}) and genotoxic effects (blood cell DSB induction measured through Comet assay). The biphasic kinetics of DNA damage observed in this species probably involves a repair mechanism,[3] for which this species may also be useful for the study of DNA damage checkpoints and damage repair mechanisms.

The evaluation of locomotory and behavioral alterations provides additional biologically and ecologically relevant end-points to evaluate sublethal effects of hazardous substances.[52,53] Despite this fact, effects of contaminants on organism locomotion are scarcely studied mainly owing to technical limitations to measure specific induced responses and the scarce knowledge of the natural behavior of many organisms.[52–54] Preliminary observations and assays of the locomotor and behavioral responses of adult frogs of *E. johnstonei* exposed to the mixture Roundup®SL–Cosmoflux®411F include the induction of erratic movements, tetanic contractions, loss of reflex, and abnormal body posture.[55] These and other (*e.g.*, alterations in locomotor performance, predator avoidance, reproductive and parental behavior, *etc.*) parameters may represent candidate end-points in further studies, but additional evaluation is necessary in order to confirm its robustness, reproducibility, and relevance for the specimen's health and fitness, as well as to improve its quantification using visual technological tools (*e.g.*, automatic recording system, video graphics analyzers, *etc.*).

This species also represents a potential model for the screening of early developmental anomalies induced by agrochemicals. Embryos of this species can be easily stained and cleared (see Meza-Joya *et al.*[46]) to assess agrochemical-induced developmental anomalies during skeletogenesis, as well as probably during organogenesis through histological plates. Other promising end-points include morphological (*e.g.*, somite, tail, limb, eye, and head) malformations and behavioral alterations (*e.g.*, disruption of typical and/or induction of atypical embryo movements) during early embryonic development. The study of other specific developmental end-points (*e.g.*, cardiovascular and neural differentiation) requires the use of a transgenic organism that expresses fluorescent markers (*e.g.*, green fluorescent protein) in the target tissues. Transgenic technology has also been successfully used in aquatic ecotoxicology to identify chemically induced target gene activation, as well to quantify relative uptake and

accumulation of chemicals in mark tissues,[56–60] providing a more advanced and integrated system for assessing the health impacts of chemicals.[61] Although mutant lines for *E. johnstonei* are currently unavailable, the development and application of transgenic frogs to terrestrial ecotoxicology represents a fruitful area of future research.

Several tests designed to assess alternative clastogenic and genotoxic end-points (*e.g.*, chromosomal aberrations, sister-chromatid exchanges, and micronuclei induction) in aquatic organisms, including amphibians,[62–65] also represent potential tools to be implemented using *E. johnstonei* as a biological model. Alternative candidate end-points related to the reproductive success of the organism include the induction of DSBs and micronuclei in gonadal cells, through Comet assay and MN test, respectively, and the induction of reproductive anomalies and pathologies on gonadal morphology (*e.g.*, induction of testicular oocytes, ovarian dysgenesis), as well as alterations in other organs through histological plates. These end-points are particularly promising when combined with traditional end-points (*e.g.*, mortality), and may lead to a more integral evaluation of the potential effects of agrochemicals on terrestrial amphibians.

Given the wide range of sophisticated molecular ''omics'' techniques available currently (*e.g.*, genomics, transcriptomics, proteomics, and metabolomics), this species may become an obvious model organism for studying the response of terrestrial amphibians to hazardous substances at the genomic-wide level (*e.g.*, identifying molecular toxicity mechanisms and responses, candidate target genes responding to pollution stresses, metabolization and regulatory pathways, *etc.*). Beyond laboratory assays, this species also represents a good model for assessing the effects of individual and cocktails of agrochemicals through outdoor experiments, such as land-based mesocosms and field enclosures. These methodological approaches are less common, but have been successfully used to examine the effects of pollutants on amphibians accounting for multiple environmental factors under more realistic conditions.[66–68] Further ecotoxicological research using *E. johnstonei* as a model organism would be a welcome addition to better understand whether and how environmental xenobiotics can affect amphibian populations.

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CHAPTER 11

The Lizard Salvator merianae *(Squamata, Teiidae) as a Valid Indicator in Toxicological Studies*

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11.1 Introduction

The saurian family Teiidae currently contains nine genera and 116 species. Currently, two species are present in Argentina, the black tegu *Salvator merianae* (formerly called *Tupinambis merianae*) and the red tegu (*Salvator rufescens*).[1] The southern Argentine tegu or black tegu is an endemic species in South America that exhibits a wide geographical distribution comprising the states of Amazonas, Para, Rio Grande do Sul, Maranaho, Pernambuco,

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Mato Grosso and Goias in Brazil; eastern Bolivia, Paraguay, Uruguay and northern and central Argentina, and the north Patagonian province of Rio Black (Figure 11.1), including both tropical and cold climates.[2] Despite the overall differences in coloration—reddish *S. rufescens* and blackish in *S. merianae*—the character that best differentiates both species, particularly when the hides are observed, is the presence of a postmental scale in *S. rufescens* and two in *T. merianae*.[3] They have a very marked sexual dimorphism: the males have larger body size, more intense color and

Figure 11.1 Area of geographic distribution of *Salvator merianae* in South America. Reprinted from L. G. Schaumburg, G. L. Poletta, P. A. Siroski and M. D. Mudry, Baseline values of Micronuclei and Comet Assay in the lizard *tupinambis merianae* (Teiidae, Squamata), *Ecotoxicol. Environ. Saf.*, **84**, 99–103, copyright 2012, with permission from Elsevier.

present sexual buttons on both sides of the cloaca, and the difference is also observable in raw and tanned hides.[4]

Salvator merianae lives in a variety of environments, including open spaces of primary and secondary forest, savannah with thorny bushes and enclosed spaces, such as tropical rain forests, riverbanks and sandy coastal areas, and is also found in disturbed habitats, including roadsides and agricultural areas.[5] Individuals of this species are large, active hunters with generalist and opportunistic feeding. Their diet depends on age; for instance, when young they consume insects, spiders, snails and fleshy fruits, thus playing an important role in seed dispersal.[6] But when they grow, the diet expands ranging from carrion, small vertebrates, small chickens and eggs both of their own species and others, also including plants and roots, and even fungi.[7] As predators, large tegus (adults) can only be threatened by large mammals, such as big cats and foxes, but also by predatory birds. Small size juveniles can be preyed on by bigger tegus, snakes, birds and mammals.[1,8]

They are considered a valuable resource for native communities in the region where they live, who take advantage of their hides, meat and body fat for medicinal purposes and for their anti-inflammatory properties for skin diseases.[9] Populations of the genus *Salvator* living in Argentina are included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Since 2007, *S. merianae* has been under management on a sustainable use program in Santa Fe province (Argentina), called ''Proyecto Iguana''. *Salvator* populations have shown the ability to resist important annual extractions that average 1.0–1.9 million individuals per year for 30 years. Tegus are an ecologically and economically important clade of lizard. In spite of their heavily exploited situation, these species are without any risk or threat based upon their distribution, abundance, and an absence of evidence of population decline. Sustainable use programs with wild species representative of the Argentine, such as tegus, have become one of the essential pillars thanks to the participation of people living with these resources. They are involved in different tasks, such as identifying nests, egg harvesting, and logistical activities, such as surveys of natural populations and reintroduction of animals in their natural environments. All these tasks are compensated for and encouraged through economic incentives; therefore, the development of these programs not only represents an important contribution to the regional economy but also a positive impact on the awareness and commitment of local people in the preservation of the resources and protection of the ecosystem that contains them.

In contrast, the imminent pace of ''consumption'' of resources caused by environmental pressure for agricultural productivity is matched with increased pollution. The growing intensity of such holdings would significantly disrupt the diversity and variability of resources.

It has been shown in laboratory conditions that pesticides used in mainstream agriculture negatively act on the reproductive cycle of alligators[10,11] and affect their survival until at least the first year of life.[12] Alterations at the

multiorgan level would lead to a decrease in reproductive rates, changes at the genetic level and defense mechanisms.[13] All these variations may influence the quantity and quality of nests, as well as in the susceptibility of animals to infection-causing agents. These trends could negatively impact on sustainable development programs with these species. The decline in the number of nests available for harvest could threaten not only the many positive aspects achieved by local people but also a potential population destabilization of these species and their environment.

The maintenance of biodiversity depends on the preservation of remnant wildlife habitats but they are in the middle of constantly expanding intensive agriculture over neighboring ecosystems. However, constant exposure to toxic chemicals over the life span of the organisms living in such areas has been speculated to have cumulative deleterious effects.[14,15] However, serious concerns continue to be voiced about potential effects from recurrent exposure to low levels of herbicides, and differential sensitivity of animals and humans in relation to age, sex or size.[16] Pesticides may produce deep consequences in ecosystems, including death of organisms and lesions on animals, suppression of the immune system, disruption of the endocrine system, reproductive inhibition or failure, teratogenic and carcinogenic effects, cellular and molecular alterations, including DNA damage.[17] Even when pesticides are present in low concentrations, they can cause non-detectable effects in some organisms, but may induce a different kind of alteration or modification, such as genetic and physiologic disorders, and in the long run reduce their life span.[18] Both laboratory and field studies have shown that sub-lethal and sub-organismal level effects, such as DNA damage and small turnover rate for proteins, influence energy metabolism, fitness and reproductive success, leading to population-level effects. Indeed, increased genomic instability has been suggested to play an important role in the decreased fitness of the populations.[19–21] In view of this, habitats adjoining croplands are under high pressure and the impact of pesticides on the associated wildlife is, in most cases, unknown.[22–24]

In the recent past, as a result of the expansion of the agricultural frontier, many areas in the natural geographic distribution of local wildlife, among them lizards and particularly the tegus, are being exposed to contaminants. Juveniles and adults may be exposed through food, water and sediments presented in the natural environment where they live. Nesting materials could be exposed to pesticides. In addition, pollutants accumulated in the mother could reach the embryo through the yolk, also affecting embryonic development *in ovo*. Frequently, female tegus build nests adjacent to crops (Figure 11.2).

For this reason, embryos are exposed to pesticides used on these crops. Embryos and hatchlings may be exposed to such compounds coming into contact with the eggshell from the atmosphere during incubation or after hatching. The period of maximum pesticide application coincides with the breeding season of this species (November to January), posing a serious contamination risk for developing embryos and neonates. Despite

Figure 11.2 (A) Picture of a *Salvator merianae* nest built at the bottom (blue circle) of a tree next to the soy crop. (B) Picture of *Salvator merianae* eggs inside the tree.

consistent calls for greater emphasis on reptile ecotoxicology research, there is still a lack of knowledge regarding the responses of reptiles to contaminants.[25]

11.2 Evaluation of Effects of Environmental Agent

The presence of a toxicant in the environment generates at least a suspicion of potential risk, however, to say that something is contaminated we need to detect the toxicant in the body, and some poisoning symptomatology or clinical changes should appear. Moreover, the relationship between the level of the toxicant in the body and the toxic response is complex and difficult to predict because it depends on many factors. A method of quantifying exposure to xenobiotics and their possible impact on the human species is the use of biological monitoring procedures using biomarkers.

11.3 A Pathway to the Truth

In some cases, environmental factors are under the control of the experimenter and studies may be designed to model real environmental conditions more closely. The important question is whether the same or similar responses are observed in the field. Even, it can obtain some approximation except when it is used in seminatural studies.

Biomarkers are suitable tools for assessing and monitoring the health of an ecosystem, considering that the consequences of exposure to xenobiotics on wildlife populations may be reflected early as changes at the cellular or subcellular level. Genotoxicity biomarkers are used to evaluate alterations to the genetic material of organisms with different consequences on cellular

function. In recent years, the most commonly selected biomarker tests for genotoxicity are the Micronucleus Test (MN) and the Comet Assay (CA) owing to their high sensitivity and low cost. They are frequently applied in ecotoxicological *in vivo* and *in situ* studies to analyze DNA damage in organisms living in contaminated environments. A lack of information about genotoxicity studies, together with environmental degradation as a result of the utilization of pesticides, led us to begin a monitoring study in the tegu lizard. Among the short-term tests applied as biomarkers of genotoxicity in wild species, the MN and CA are preferred methods owing to their sensitivity for detecting chromosomal and DNA damage induced by physical and chemical agents at an early stage, the possibility to apply them in any nucleated cell type, and the small sample required.[26,27] The sensitivity of these tests may be compared although both methods measure different endpoints and, as a result, they complement each other.[28,29] We have adapted techniques for genotoxicity evaluation and determined baseline DNA damage in peripheral blood erythrocytes because a few studies have successfully applied the MN test and CA (Figure 11.3) in reptile erythrocytes to determine basal values of DNA damage,[30,31] and for *in vivo* evaluation of genotoxicity induced

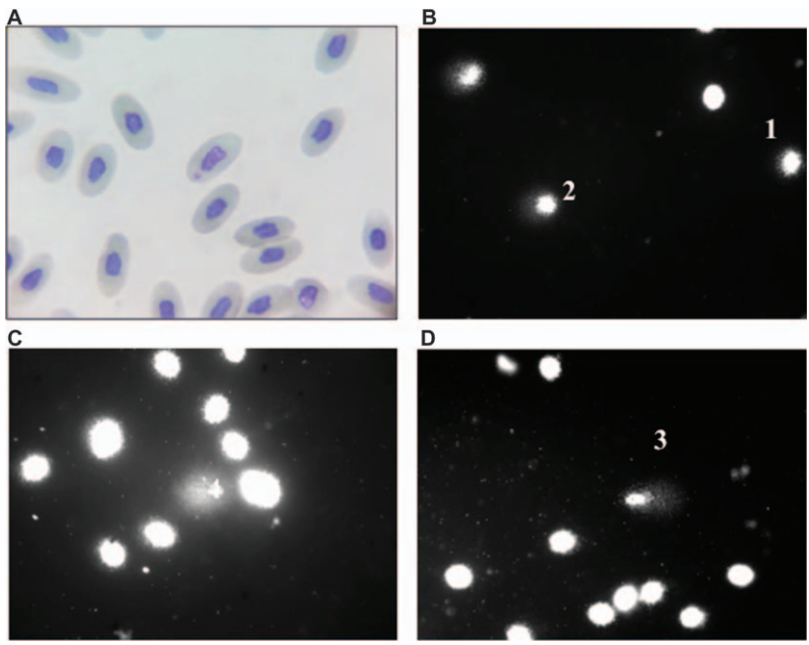

Figure 11.3 (A) Erythrocytes of *S. merianae* showing the presence of Micronucleus (Giemsa 1000×); (B), (C) and (D) Nucleoids with diferents categories of damage from 1 to 4 (400×).
Reprinted from L. G. Schaumburg, G. L. Poletta, P. A. Siroski and M. D. Mudry, Baseline values of Micronuclei and Comet Assay in the lizard *tupinambis merianae* (Teiidae, Squamata), *Ecotoxicol. Environ. Saf.*, **84**, 99–103, copyright 2012, with permission from Elsevier.

by physical[32] or chemical agents.[12,33] In all cases, they demonstrated a high sensitivity for detecting the effects of genotoxic agents.

11.4 Goals of Biological Monitoring

Some considerations are very important to avoid interference that can be interpreted as normal and that may have implications at the outset. All of these facts can be reduced if we determine the baseline values of DNA damage and, in this way, we propose the tegu lizard as a sentinel species to characterize genotoxic effects in natural environments.

Tegus were divided into laboratory tests and seminaturals studies, where one stops controlling certain variables and then progressively monitors exposure and effect. Within each type of exposure, different stages of development were taken. For both studies under controlled and semi-controlled conditions, in appropriate cases it is necessary to adapt or simulate the system so that data can be interpreted in a more approximated or realistic way. The tests performed in the laboratory or under controlled conditions never start from ''zero'' and must be referenced in many cases as a baseline. This consideration is very important to avoid interference that can be interpreted as normal. In the case of genotoxicity, all of these facts can be reduced if we determine the baseline values of DNA damage and, in this way, it is very useful to propose an animal as a sentinel species to characterize genotoxic effects in the natural environments.

The baseline values are very useful as reference values for future studies to assess the effects of some contaminant agents in *S. merianae*. Grisolia *et al.*[34] detected that different organisms have differential sensitivity observed in the baseline DNA damage in species living in the same environment, and may also be affected by high inter-individual variability.[35] Udroiu[36] indicated the presence of spontaneous MN influenced by intrinsic factors like mechanisms to remove old or damaged erythrocytes. Ectothermic organisms have a low metabolic rate and have been demonstrated to be more sensitive to the effects of xenobiotics and their recovery may be slower than that of other non-reptilian species.[37] In this context, the basal level of DNA damage has been shown to be influenced by multiple factors and all studies should consider variables like species, sex, and age.[38] The level of DNA damage also varies depending on the species and the cell type used, and this will influence the sensitivity of the assay to detect genotoxic effects.[39]

Micronucleus baseline values were reported for two species of lizards, *Iguana iguana* and *Ctenosaura pectinata* (BFMN 0.10 and 0.05/1000, respectively),[30] and for other reptilian species, such as snakes, turtles and crocodiles (BFMN from 0 to 0.30/1000).[38] Similar results were obtained for the snake *Hierophis gemonensis* (0.30/1000)[40] but in *S. merianae*, the BFMN was lower (0.957 ± 0.27) (Table 11.1). Our results coincide with those reported by Poletta *et al.*[41] for *C. latirostris* both in BFMN (0.87 ± 0.74) and BDI (103.40 ± 73.36), and furthermore, we found no relationship with size or sex of *S. merianae*. Those differences among studies could be explained by

Table 11.1 Basal frequency of MN and baseline index values for *S. merianae*.

Animal	Sex	BFMN[a]	BDI[b]
1	♂	5	116
2	♀	0	102
3	♂	1	110
4	♂	1	102
5	♂	2	111
6	♂	1	100
7	♂	0	101
8	♀	2	108
9	♂	2	102
10	♂	1	104
11	♂	0	100
12	♀	0	104
13	♂	0	105
14	♀	1	100
15	♀	0	102
16	♀	1	104
17	♀	1	100
18	♀	1	102
19	♀	0	100
20	♀	0	104
X ± SD		0.95 ± 0.27	103.85 ± 0.97

[a]Basal frequency of Micronucleus (MN/1000 cells counted).
[b]Baseline damage index (determined by the analysis of 100 comet images classified in arbitrary units).

different analyzers, low number of individuals, conditions of animals, different times of enclosure, management *etc*.

During recent years, several wild species from different orders have been used as bioindicators in the monitoring of environmental quality,[42–44] taking into account that their susceptibility to different xenobiotics can be used as an early warning of environmental alterations.[45] Based on some results, *S. merianae* was demonstrated to be a good indicator to evaluate the effects of xenobiotics through the genotoxicity assessment (MN and CA). Besides, the possibility to work in frames of wild species under management, as is *S. merianae*, implies numerous advantages plus it is easy to obtain blood samples without harmful mechanisms, positioning the species in prime place for future studies. Currents studies are trying to determine if this species could be considered as a sentinel organism *in situ*. Preliminary results obtained from its home range confirm the sensitivity of the MN and CA tests to be applied as biomarkers of genotoxicity in erythrocytes of *S. merianae* and allow them to be proposed as bioindicators in the monitoring of environmental quality.

On the other hand, MN and CA baseline values were found to be independent of the clutch of origin, sex and size of the animals, showing that they are quite stable among tegus, and thereby demonstrating the suitability of these techniques as accurate monitoring tools for the evaluation of

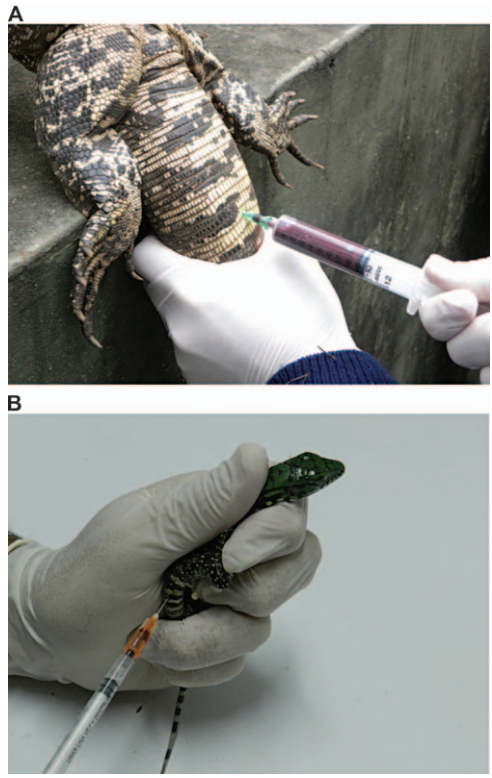

Figure 11.4 Methods to take blood from *S. merianae* of different sizes and and the dificulties obtaining large volumes from neonates.

genotoxic agents, as well as the suitability of this species as a sentinel organism of genotoxic effects. This was the first reference to the application of genotoxic techniques in *S. merianae*. Moreover, the possibility to apply them in peripheral blood erythrocytes and the fact that blood collection in this species does not cause any damage to the animals means they represent great advantages as non-destructive monitoring techniques (Figure 11.4).

11.5 Studies *In Ovo*

Over the past 50 years, the expansion and intensification of agriculture have triggered environmental problems at different scales and levels, constituting the most persistent anthropic activity during this time. Inevitably, the ecosystem transformations have induced a nonreversible loss of habitat and biodiversity, and disruption of the structure and functioning of ecosystems; this demonstrates one of the most prominent effects of the explosion in the use of transgenic soy and implementation of new agriculture technologies in tropical and subtropical regions.[46] In Argentina, major threats to biodiversity include deforestation and the draining of marshes in order to

allocate more land to agriculture, particularly to soybean crops.[47] This current agricultural model is directly associated with the high usage of pesticide formulations. The formulations are mixtures made with various active substances (the main component responsible for killing effects) with other ingredients called adjuvants or surfactants, which are ingredients included in formulations to increase the adsorption and improve the effectiveness of their action. Both are present in high percentages in some formulations and are considered inert ingredients, although in many cases they exceed the toxicity of the active ingredient.[12,48] This kind of agricultural activities imply the use of commercial glyphosphate-based formulations, which are complex and variable chemical mixtures, rather than the use of the active chemical ingredient (glyphosate) alone. Surfactants may have a toxicity several times higher than glyphosate itself, making the formulated product of greater toxicity than the active ingredient (*a.i.*).[49–51] One of the most widely used commercial glyphosate formulations is Roundup®, particularly in applications involving plant varieties that are genetically modified to tolerate glyphosate treatment.[52,53] In addition, in cultivated soils of Southeast Buenos Aires (Argentina), glyphosate was detected in a concentration of 1502 $\mu g\,kg^{-1}$ one day after the last application and it was still present in the environment 190 days later, at a concentration of 35 $\mu g\,kg^{-1}$.[54] The temporal variation of glyphosate levels depends directly on the moment of application and the frequency and intensity of rain events,[55] and it is very frequent to detect some effects on non-target species because of cumulative residues in the environment.

One of the potential risks is *in ovo* pesticide exposure in the natural environment. Based on that, toxicological assays were conducted in which we exposed tegu eggs under controlled conditions to increasing concentrations of the glyphosate formulation Roundup® (from RU50 to RU1750) by topication (chemical applied directly to the eggshell) at the beginning of the incubation period.

Some eggs from three clutches were harvested in the managed natural reserve "El Fisco" (30° 11′ 26″ S, 61° 0′ 27″ W; Figure 11.5), an area free from agricultural activities or any other source of contaminants, located in Santa Fe province, Argentina.

After collection, eggs were immediately transported to the lab facilities (Lab. Zool. Aplicada: Anexo Vert., FHUC—UNL/MASPyMA). Eggs were measured in length, weighed and then placed in plastic trays with wet vermiculite as an incubation substrate. Then they were randomly assigned into eight experimental groups of 12 eggs each (six eggs per replica) (See Table 11.2). At the beginning of the incubation, 25 $\mu L\,egg^{-1}$ of pesticide at different concentrations (depending on each treatment) was applied topically (Figure 11.6).[56]

After that, the eggs were covered with wet vegetal material in order to mimic the natural conditions of incubation in a wild nest. Artificial incubation was carried out for a period of 60 ± 5 days under controlled temperature (29–31.5 °C) and humidity (30%). A pesticide solution was

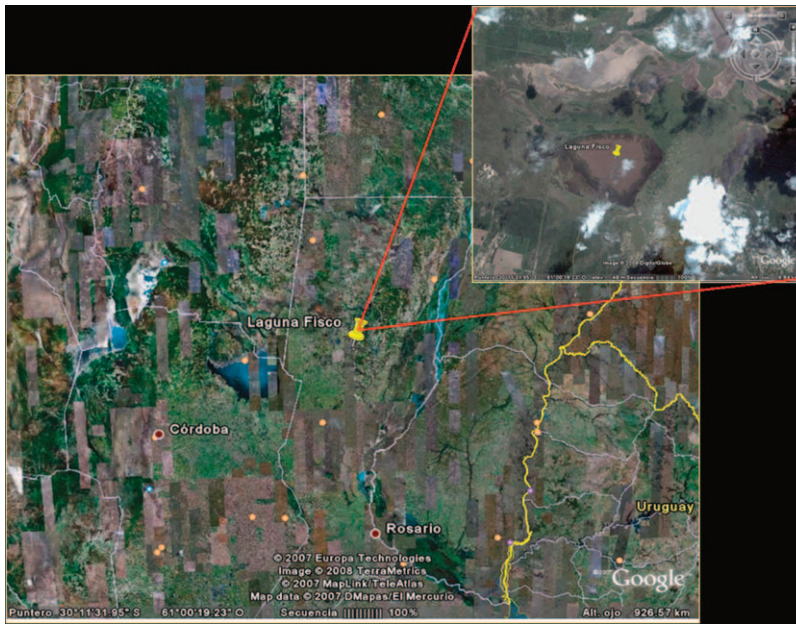

Figure 11.5 Satellite image of managed natural reserve ''El Fisco''. Area free of agricultural activities or any other source of contaminants, located in Santa Fe province, Argentina.
Reprinted from L. G. Schaumburg, P. A. Siroski, G. L. Poletta and M. D. Mudry, Genotoxicity induced by Roundup® (Glyphosate) in tegu lizard (*Salvator merianae*) embryos, *Pestic. Biochem. Physiol.*, **130**, 71–78, copyright 2016, with permission from Elsevier.

Table 11.2 We used 96 eggs from three nests randomly distributed according to the following table and artificially incubated for a period of 60 ± 5 days at 29 ± 0.5 °C.

Experimental group	Treatment	Concentration
Positive control (CP)	Ciclofosfamide	400 μg egg^{-1}
Negative control (CN)	Destilled Water	25 μl (vol.)
RU50	Roundup®	50 μg egg^{-1}
RU100	Roundup®	100 μg egg^{-1}
RU200	Roundup®	200 μg egg^{-1}
RU400	Roundup®	400 μg egg^{-1}
RU800	Roundup®	800 μg egg^{-1}
RU1600	Roundup®	1600 μg egg^{-1}

made from the commercial formulation Roundup® Full II (66.2% glyphosate as active ingredient, *N*-(phosphonomethyl)glycine monopotassium salt; Monsanto Co., Argentina) in distilled water. Then, serial dilutions of it were made to obtain the topical Roundup® doses. Owing to the lack of previous data for genotoxicity evaluations of this formulation on *S. merianae*, we used concentrations based on data from a previous study on other reptilian

Figure 11.6 Mecanism of applying contaminant over the eggs under controlled conditions called topication. By this method we can evaluate the effects of exposure to contaminants on the development of tegu embryos.

species[12] and adjusted for the average egg weight of the tegu lizard (20 g). In order to determine the effective dose range for this formulation in tegu lizard, a wide range of concentrations (50–1600 μg Roundup® egg^{-1}) were tested. Taking into account that Roundup® FULL II contains 66.2% glyphosate and its recommended field application rate is 2–3 L ha^{-1}, the resulting applied concentration is approximately 1300–2000 g ha^{-1}. Moreover, differences in the percentage of DNA damage increment related to negative control observed between the highest concentration of glyphosate applied (RU1750) and the positive control (cyclophosphamide, potent genotoxic agent use) were fairly slight.[12]

Immediately after hatching, whole blood samples were obtained from the caudal vein, measured in snout–vent length (SVL) and weighed. Hatching success and presence or absence of external body abnormalities were registered. Genotoxic effects were assessed in the newborn tegus later (3 and 12 months) using the MN test and CA in erythrocytes.

In recent years, several studies have described the presence of nuclear abnormalities (Nas), which are also considered to be induced by genotoxic agents. The NAs assay was applied according to the criteria of Carrasco *et al.*[57] The frequencies of NAs were calculated from 1000 mature erythrocytes per animal (in two slides), considering the following categories: nuclear bud (NB), notched nuclei (NN), eccentric nuclei (EN) and irregular nuclei (IN). In addition, the presence of anucleate (AE) erythrocytes was observed (Figure 11.7). Results are expressed as the frequency of each category and total NAs (sum of all the observed NA).

The results revealed genotoxic effects of Roundup® evidenced by the MN test and CA in erythrocytes of *S. merianae* exposed *in ovo* and *in vivo* and agreed with similar studies. Cavalcante *et al.*[58] observed positive results only with the Comet Assay and no statistically significant increase in the FMN and NAs in erythrocytes of the fish *Prochilodus lineatus* exposed to RU (10 mg L^{-1}). Similarly, other studies that evaluated the genotoxic effect of

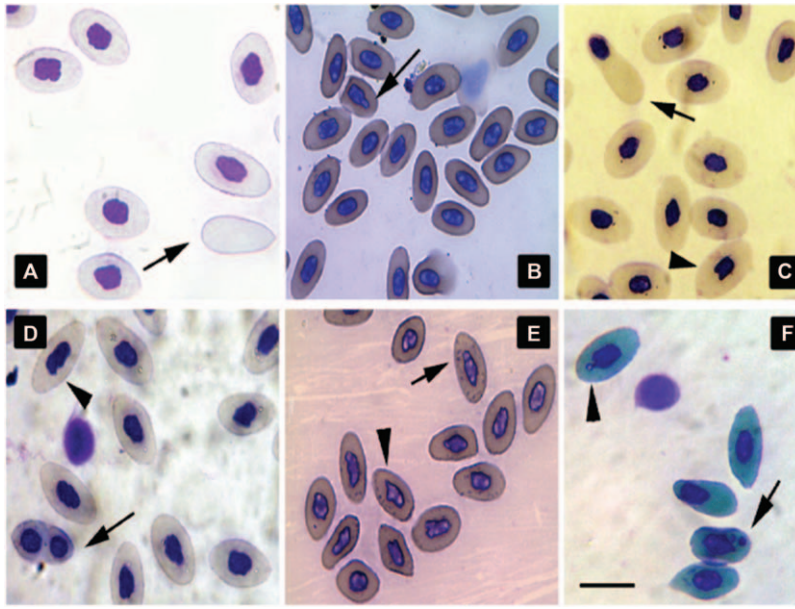

Figure 11.7 Erythrocytes of *S. merianae* showing the presence of: (A) AE: anucleated erythrocyte (long arrow), (B) NB: nuclear bud, (C) EN: eccentric nuclei (long arrow) and, NN: notched nuclei (short arrow), (D) BIC: binucleated cell (long arrow) and NN: notched nuclei (short arrow), (E) NB: nuclear bud (long arrow) and IN: irregular nuclei (short arrow), (F) MN: micronucleus (long arrow) and NB: nuclear bud (long arrow) (1000×). Reprinted from L. G. Schaumburg, P. A. Siroski, G. L. Poletta and M. D. Mudry, Genotoxicity induced by Roundup® (Glyphosate) in tegu lizard (*Salvator merianae*) embryos, *Pestic. Biochem. Physiol.*, **130**, 71–78, copyright 2016, with permission from Elsevier.

6.67 $\mu g\,L^{-1}$ Roundup® on the fish *Corydoras paleatus* at 3, 6, and 9 days of exposure did not show differences in MN frequencies between groups and periods, but CA showed a high rate of DNA damage in the group exposed for all treatment times.[59] Our previous studies performed in *C. latirostris* (*in ovo* exposure) found a positive relationship when Roundup® concentrations increased, showing an increase in DI and MN frequencies in erythrocytes, reinforced by enzymatic and metabolic alterations.[12,60] Similarly, Cavas and Könen[61] found a significant dose-dependent increase in the FMN, NAs and DI at 48, 96 and 144 h after exposure to 5, 10 and 15 ppm of Roundup® in the fish *Carassius auratus*. On the other hand, Guilherme *et al.*[62] demonstrated that after one day of exposure to 58 and 116 $\mu g\,L^{-1}$ Roundup® concentrations, the frequencies of NAs on erythrocytes of the fish *Anguilla anguilla* were not different, but there was an increase after three days of exposure compared with the control, and an increase of DI at both times, although it was stronger at one day of exposure.

The detection of MN together with other NAs in erythrocytes provides an index of accumulated genetic damage during the life span of the cells.[63]

Therefore, MN frequency in peripheral erythrocytes is the result of the dynamic balance between the formation of micronucleated cells and their elimination.[64] Accumulation of DNA damage may occur either through an increase in the number of DNA-damaging events or owing to a decrease in DNA repair capacity and/or antioxidant system.[65] In this respect, other authors have observed that after the end of an exposure to glyphosate formulations, DNA damage still increases.[61] DNA repair is compromised after exposure to high levels of Roundup® constituents/metabolites because they generate a certain inhibition of reactive oxygen species (ROS), identifying another interesting point of risk associated with this tested chemical.[65] In addition, the generation of ROS is implicated as a mechanism of Roundup® toxicity in larval amphibians.[66] A recent molecular modeling study has evaluated the toxicity of Roundup® through the interaction with the binding site of mitochondrial succinate dehydrogenase, inhibiting its activity.[67] On the other hand, a study that evaluated the effect on the embryology of sea urchins demonstrated that glyphosate formulations induce a delay in the kinetics of the first cell cleavage in this species owing to its action on the G2/M transition through the mobilization of the DNA-response checkpoint as a consequence of interference with DNA replication during the S phase.[68] These findings agree with others that reported some positive genotoxic results with Roundup® formulations, suggesting that this herbicide may not be as safe as previously thought, at least as a formulated product, because surfactants added to the active ingredient may considerably increase its toxicity.[61,69,70]

11.6 Studies *In Vivo* under Controlled Conditions

Life stories show particular roles in important and diverse food chains crucial for the proper functioning of ecological processes.[71] In order to mimic actual model crops, we tried to simulate a ''real'' situation when tegus are directly exposed to pesticides in surrounding environments. The evaluation of sublethal effects through the simulation of an environmental exposure can be a useful tool for studying the impact of pesticides on organisms in closer to the natural environment. The aim of this study was to evaluate the genotoxicity in juvenile tegus after exposure with a mixture made of cypermethrin (Atanor®), endosulfan (Galgofan®) and glyphosate (Roundup® Full II) in semi-natural conditions considering the same manner as used in field applications.

Animals used in this study came from eggs that were collected in the Managed Natural Reserve ''El Fisco'' (30° 11′ 26″ S, 61° 0′ 27″ W), Natural protected area (Law 12,930, 2008), Dept. San Cristobal, Santa Fe, Argentina. Prior to experimentation, the animals were randomized into two sites (Figure 11.8), with 30 animals ($N = 60$) each, and kept there for a week to acclimate to the new place.

One of the enclosures (RT) was exposed to two applications of pesticides: first with a formulation of glyphosate and the second with a mixed

Figure 11.8 Enclosures where animals were exposured to pesticides according to the application schedule used in agricultural practices in the region. Animals (from 12 to 24 months old) were acclimated for 10 days before the spread. They were removed for fumigation and then returned for 3 months.

formulation of glyphosate– cypermethrin–endosulfan equivalent to concentrations applied in the field and following the schedule established by the Instituto Nacional de Tecnología Agropecuaria (INTA) for agricultural practices in the region.[72] The control enclosure (RC) received no exposure to any pesticide, but it was treated only with water in both instances of applications. The experiment lasted 3 months. Each enclosure had two or three shelters with abundant grass, a feeder, a water fountain and the roof was covered in parts to generate ventilated shade. Before and after the experiment, morphometric measurements of all individuals were recorded to evaluate the growth of animals. At the end of the study, peripheral blood samples were taken from all animals by venipuncture of the coccygeal caudal vein and MN, NA and EC tests were applied to assess damage to the genetic material by such exposure.

A significant frequency in both the MN and the genetic damage index in the RT individuals compared to the RC individuals (Figure 11.9) was observed, but no differences were found in the frequency of AN individually or in total ($p > 0.05$) (Figure 11.9). There were no statistically significant differences in the LHC or weight of tegus between the RC and RT groups ($p > 0.05$), which is an expected result if one considers that the increase in size in reptiles is slower than in other vertebrates and requires more time so that it can manifest. Furthermore, no differences between individuals from different nests ($p > 0.05$) were observed.

This work shows that, under the conditions evaluated, pesticides have affected the genetic material, constituting a genotoxic risk to wild *S. merianae* populations living in areas permanently exposed to these chemicals. Even though this experimental design could be considered extreme or unreal, it is a fact that habitats neighboring croplands receive recurrent and continuous exposure to low levels of pesticides, which could have cumulative deleterious effects on animals living there, especially

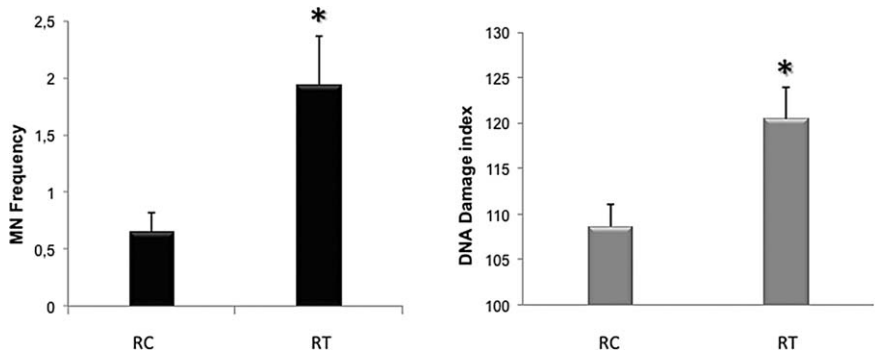

Figure 11.9 Genotoxic damage measured in erythrocytes of *Salvator merianae* exposed to pesticides in semi-natural conditions. * Indicates statistically significant difference in RT compared to the RC (Mann–Whitney test, $p < 0.05$).

considering the differential sensitivities that exist among them. In this context, there is a need to temporarily track tegus environmentally exposed to pesticides for their evaluation and characterization of the genotoxic effects observed in long term.

11.7 Genotoxic Evaluation of Tegu Lizard Environmentally Exposed to Pesticides

The natural habitat of the tegus in the Santa Fe province is fragmented owing to agricultural expansion, so the populations of *S. merianae* abound and nest on the periphery of cultivated areas. Nesting sites are restricted to the few remaining areas of vegetation in these environments. The breeding season of the iguana overlaps with the period of application of agrochemicals in agricultural areas, including glyphosate formulations, cypermethrin and endosulfan, which increases the risk of exposure for iguanas at all stages of life. Given that exposure to these contaminants can influence the DNA integrity of an organism and, therefore, affect their development,[73] it is important to evaluate the effects of these agrochemicals on the health status of the animals in their ecosystem. The detrimental effects on hatching may alter the survival of populations that inhabit areas adjacent to the crops and are frequently exposed to agrochemicals, modifying the local biodiversity and the ecological equilibrium. More research on the potential genotoxicity of this and other agrochemical formulations is needed to adequately assess the risk to which *S. merianae* is really exposed.

The aim of this study was to evaluate the genotoxicity in neonate and adult *S. merianae* exposed environmentally to agrochemicals often used in agricultural activity, using the MN test, CA and the occurrence of nuclear abnormalities.

Individuals (young and adults) used in this study were obtained from different districts of the province of Santa Fe, in the margins of plots

cultivated with soybean (exposed) and in remote areas of crops with a distance of more than 1000 meters (not exposed). At each sampling, the nests were marked with GPS and eggs were incubated in the wild under the care of the female parent. Close to the end of the incubation period, the eggs were harvested and transferred immediately to the lab facilities. Upon hatching, the neonates were weighed and measured, then blood samples were taken to implement the MN test, EC and AN, in order to evaluate the genotoxic effect induced by chemicals in wild individuals environmentally exposed.

Adult animals ($n = 14$) were caught during the breeding season from two different areas: (1) on the periphery of cultivated (five females and two males) fields and (2) others without exposure, in the Managed Nature Reserve ''El Fisco'' (six females and one male). All animals were measured in length, blood samples were taken, and then they were released.

Neonates came from eggs harvested during the breeding season from nests located on the periphery of the cultivated fields at a distance of approximately 3 m from the front lines of soybean culture. Eggs from nests identified in both areas were incubated in nature for a while and then harvested to complete the gestation period (60 ± 5 days) maintaining a controlled temperature of 30.5 ± 0.5 °C. After hatching, they were measured in length (LHC) and weight, and blood samples were taken from the tail vein.

MN test and CA were applied to the neonate and adult blood samples based on the specific changes for this species,[74] and AN according to the protocol of Carrasco *et al.*[57] The AN were counted as core notched (NM), nuclear buds (BN), irregular nuclei (NI), eccentric core (NE), and counted the total nuclear abnormalities (ANT: NM + BN + NI + NE).

Our results show a statistically significant difference in the rate of DNA damage, frequency of MN and the nuclear bud category of AN in erythrocytes of neonates exposed in respect of the area control ($p < 0.05$).

In addition, statistically significant differences in the frequencies of MN and AN (BN) and ID among different nests used ($p < 0.05$) were observed. By contrast, in adult animals no differences in the frequencies were found in MN and AN between the exposed group and the unexposed group, nor between males and females ($p > 0.05$).

Simultaneous expression of morphological abnormalities of the nuclei and induction of MN have received considerable attention as biomarkers of clastogenicity, as an association between the frequency of injuries and exposure to genotoxic agents[61] was observed. These results support our hypothesis about the risk of environmental exposure of *S. merianae* to agrochemicals in their natural habitat, especially for newborns who showed genotoxic damage in their blood cells. It is important to realize that early exposure to these chemicals and non-repair of DNA damage can trigger a cascade of biological consequences affecting the ontogeny of organisms.

In recent years, the number of studies on the biological effects of pesticides has increased; however, sometimes there are contradictory and controversial results about their genotoxicity. They can join with a high variety of

biomolecules, including DNA. In addition, they induce ROS formation, which may be involved in the production of DNA-single strand breaks.[75,76]

To fully understand the impact of a contaminant under field conditions it is important to also consider potential impacts of natural stressors. Reductions in habitat quality have been related to changes in invertebrate communities and consequently in food availability for vertebrates.[77] Even if some cases could be more favorable than others, all fields supported arthropod communities sufficient to sustain these subpopulations.[78]

Lack of information about genotoxicity studies, together with environmental degradation as a result of the utilization of pesticides, led us to begin a monitoring study in the tegu lizard. We adapted techniques for genotoxicity evaluation and determined baseline DNA damage in peripheral blood erythrocytes. Then, we used these biomarkers to evaluate the effects induced by embryonic exposure to commercial glyphosate formulations in laboratory-controlled conditions, as well as the effects of pesticide mixtures under semi-natural exposure conditions. At this moment, we are evaluating populations of tegu lizards environmentally exposed in their natural habitat to different agrochemicals used in soybean, maize, and other crops.

Data provided here will be useful for future work involving the biomonitoring of natural regions where *S. merianae* can be under increasing contaminant pressure, mainly taking into account the increasing ecological and economic value that this species has.

In our study, the pesticides were tested as the complex commercial mixtures as this is the form in which they are routinely applied in agriculture and introduced into the environment. Considering the wide use of these pesticides, mainly for agricultural activities, risk assessment of commercial technical formulations regarding their potential for promoting damage in the genetic material of natural species has to be considered of primary importance.

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CHAPTER 12

The Terrestrial Lizard Podarcis sicula *as Experimental Model in Emerging Pollutants Evaluation*

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12.1 Introduction

Over the past few decades a lot of evidence has suggested that many environmental chemicals, changing the physiology of the endocrine system, can alter growth, reproduction and survival of the exposed species.

Organisms of both terrestrial and aquatic food chains accumulate certain pollutants to the extent that they may become toxic.[1–3] Most investigations are focused on the aquatic food chain and little is known in this field on terrestrial vertebrates.[2,4–7]

Amphibians and reptiles are considered valuable indicators of local contamination since they are more sensitive to the effects of pollutants than birds or mammals.

Until 10 years ago, ecotoxicological studies on reptiles were very scarce and a large gap in environmental contamination effects for this group was

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recorded. However, recently the scientific literature in this field has become more enriched thanks to the studies performed on the lizard *Podarcis sicula*.

Lizards are a cosmopolitan group of terrestrial, fossorial, herbivores and insect predators, characterized by strong site fidelity, spending their entire life within small home ranges.[8] They are also an important component of terrestrial ecosystems as well as an integral part of food chains constituting an important link between invertebrates and predatory vertebrates as well as other reptiles, birds and mammals.[9] Since the adult specimens are secondary or tertiary predators, they are susceptible to bioaccumulation of persistent environmental pollutants and are good bioindicators of environmental contamination. They are able to bioaccumulate and biomagnify pollutants to the same extent as birds and mammals.[10,11] In addition, since the lizards mainly eat insects, it must be considered that insecticides and pesticides may have the greatest impact on lizards with respect to other top predators. For all these reasons, lacertids are considered good sentinels of the quality of terrestrial habitat in a growing number of studies of environmental biomonitoring.[12]

12.2 Who is the Lizard *Podarcis sicula*?

The Italian wall lizard *Podarcis sicula* (Figure 12.1) is an oviparous species that ranges throughout Italy south of the Alps, including on Sicily, Sardinia, and many other islands in the Tyrrhenian Sea. However, *P. sicula* specimens are also present in Corsica, southern France, the Iberian Peninsula and along the Adriatic coastal area, preferring a temperate climate. Its reproductive cycle is annual and is dependent on temperature and photoperiod.

The female lays eggs in two or four ovulatory waves, between April and July (breeding period). During the annual cycle, the oocytes undergo a slow phase of growth (from August to March) followed by a short and fast vitellogenic phase lasting 20–30 days during which three to four oocytes, recruited in each ovary at each ovulatory wave, accumulate a large amount of yolk reaching the ovulatory size.[13] During the breeding period, both small white previtellogenic oocytes and big yellow vitellogenic oocytes, ready to be ovulated, are present in the ovaries.[14] In this period the liver, stimulated by

Figure 12.1 *Podarcis sicula*, probably during copulation.

estradiol-17β secreted by the ovaries, synthesizes vitellogenin (VTG), the major precursor of the yolk proteins.[15,16] Furthermore, in *Podarcis* the presence in the liver of the α type of the estrogen receptor (ERα) is an essential prerequisite for the occurrence of VTG synthesis.[17] Subsequently, VTG released into the blood reaches the theca of the ovarian follicles and, through the intracellular space of the granulose cells, is taken up into the oocytes by micropinocytosis. The micropinocytotic vesicles merge with each other and give rise to the primordial yolk platelets that become mature yolk globules.[18,19] Inside them, VTG is proteolytically cleaved into lipovitellin and phosvitin by cathepsin D.[15,20] In all the oviparous vertebrates, lipovitellins and phosvitins represent more than 80% of the total yolk proteins in the oocytes and constitute the primary source of nutrients, such as lipids, amino acids, carbohydrates and phosphates during embryo growth.

Podarcis sicula females show an average of about six to eight eggs per clutch; eggs are deposited under rocks, soil and debris. No maternal care is present. The optimal incubation temperature for developing embryos is about 26 °C; embryos show a very narrow temperature range in which development can occur normally.[21] Incubation lasts about 50–60 days, depending by temperature; at birth the offspring are about 4 cm in length and have the same morphology as adults.

In *Podarcis* males the intense spermatogenic activity takes place in spring. In this period, the spermatozoa produced in the testis pass through the rete testis and then into the epididymal channel to be subsequently ejaculated.[22,23] In the early summer, the spermatogenesis is over and males enter in a status of refractoriness considered a condition of physiological hypophysectomy, during which they are unresponsive to endogenous or exogenous hormones.[24] In the fall, *Podarcis* males show a spermatogenic recrudescence that has been considered reminiscent of two reproductive events probably present in the ancestor of this lizard living in a milder climate environment.

The epididymis allows the survival, viability and storage of spermatozoa. In *Podarcis* the epididymis is regionalized to an initial segment called *caput* that comprises the *efferent ductules*, followed by the middle and terminal segments, respectively termed *corpus* and *cauda*. *Podarcis* epididymis is characterized by a cyclic secretion that occurs in the *corpus* during the mating period when this compartment displays a cylindrical epithelium producing a great amount of secretory granules released into the enlarged lumen, where many spermatozoa are also present. In the winter stasis, the epithelium is cuboidal and non-secreting and surrounds the small lumen, totally empty of granules and sperms.[25]

12.3 Pollution by Organic Contaminants with Estrogen-like Action: Fertilizers and Manure

Emerging pollutants that arouse particular concerns are organic molecules that can interfere with the health and welfare of the live organisms. Among

these molecules, particular attention is given mainly to the Endocrine Disruptor Chemicals (EDC) that, interfering with the physiology of the endocrine system, cause adverse effects in animals and humans. Unfortunately, the list of EDC is considerable and includes chemical substances of wide use, so nowadays EDCs are widespread in the environment. In particular, estrogen-like compounds originate from a variety of sources, are present as the co-formulant of pesticides (alkylphenol polyethoxylates) and, in the form of steroidal hormone metabolites, can also be present in the manure used for fertilization of soils dedicated to organic farming.[26,27] The main problem arising from the use of compounds with estrogen-like activity is their molecular similarity with estradiol-17β (E2): mimicking the E2 action, they compete for the ligand binding site of the estrogen receptors (ERs),[28,29] then, the receptor–EDC complexes bind to chromatin in the cell nucleus, stimulating the synthesis of specific RNA and proteins.

Among alkylphenols, nonylphenol (NP) is by far the molecule with greater commercial interest: it is used to produce surfactants for a wide variety of applications and consumer products, such as emulsions for pesticides, the paper, textile and leather industries, paints, adhesives, inks, washing agents, care products, cleaners and detergents. The main issue with the use of NP is that it is lipophilic, is retained in the sediments where it is persistent with a half-life of more than 60 years and is bioaccumulable in vegetal and animal tissues.[30–34] It is widely known from *in vivo* and *in vitro* studies that NP acts as an estrogen-like compound; however its estrogenic potency is lower than that of natural estrogens. In addition, the binding affinity of NP to ERs is estimated to be approximately 4.0×10^{-5} lower than those of natural estrogens.[35–40]

The worms accumulate NP through the ingestion of polluted sediments and the organisms placed on the top of some trophic chains could be affected by the presence of NP in their food.[41,42] Human exposure may also occur by ingestion of contaminated water and in foods such as vegetable crops, milk and meat.[43]

To limit the presence of substances such as NP in food, in recent years organic agriculture practices have greatly increased, where chemical substances commonly used as pesticides and fertilizers are banned. Indeed, organic farming allows exclusively the use of natural derivates as fertilizers [Council Regulation (EC) No 834/2007 and Commission Regulation (EC) No 889/2008]. The manure produced in considerable quantities within the same farms is the most widely used organic fertilizer. It consists of the solid and liquid manure of livestock mixed with materials of various origins that form the animal litter. The animal litter may constitute several materials, such as straw, corn stalks, peat and wood sawdust. The choice of the litter can strongly influence the characteristics of the manure since these substances differ not only in their chemical composition but also their imbibition ability (important to retain the urine). Furthermore, the composition of manure varies depending on the species, age, sex, state of health and feeding of the animal producers.

The main problem with the use of the organic fertilizers is that the urine and faeces of farm animals of all species and sex are the main route of estrogen excretion in the environment. Livestock expel mainly 17α-estradiol, 17β-estradiol and estrone. Generally, hormones are excreted in conjugated, biologically inactive form but they may be converted into active non-conjugated forms through the action of bacteria such as *Escherichia coli*.[44]

12.4 Pollution by Heavy Metals: Cadmium

It is emerging that metal ions are also capable of interfering with estrogen action, so defining a class of inorganic xenoestrogens now termed metalloestrogens.[45] Among metals, various effects of cadmium ions (Cd^{2+}) on reproductive endocrinology have been described.[45,46] Cadmium is a widespread heavy metal continuously introduced into the atmosphere and soil through the smelting of ores, burning of fossil fuels, waste incineration, and urban traffic. It is also a by-product of phosphate fertilizers. Because of its marked bioaccumulation, Cd concentrations in manure can be very high, exceeding the allowed values for agricultural soils.[47] The primary routes of cadmium exposure in terrestrial animals are *via* inhalation and ingestion of cadmium-contaminated food.[48] As a non-essential trace element, cadmium may cause toxicity by disturbing the cellular homeostasis of essential metal ions, such as copper, zinc, and calcium; it is also able to displace these metals from pre-existing complexes.[49] Intracellular damage caused by cadmium exposure includes protein denaturation, lipid peroxidation, generation of reactive oxygen species and DNA strand breaks; preferentially accumulated in the liver and kidneys, Cd ions can be found in all tissues.[50–52]

To prevent cellular damage, cells respond to Cd exposure by increasing the expression of metallothioneins (MTs), cysteine-rich, low molecular weight (6–7 kDa) metal-binding proteins considered to be key molecules involved in biological processes related to the metabolism of essential and toxic metal ions.[53] MTs provide a mechanism by which the metal can be sequestered in a relatively inert, non-toxic state.[54] The first measurable effect of heavy metals in the cell is the synthesis of MT mRNA. The inducibility of MTs by heavy metals and the increase of MT mRNA levels have been utilized for monitoring environmental pollution in biological specimens collected in the field.[21,55] In recent years, the improvement of analytical techniques and the increased understanding of the mechanisms of MT induction have strongly encouraged the use of MT as an environmental marker for metal pollution, mainly in aquatic organisms[56,57] and terrestrial invertebrates;[56,58] data on the distribution of MT in tissues of wild terrestrial vertebrates exposed to Cd contamination are still limited.

12.5 *Podarcis sicula* as Sentinel Lizard

In recent years, we have used *Podarcis sicula* specimens caught in rural, suburban and anthropized areas to monitor soil pollution.

The principal approach that we used to monitor soil pollution by estrogen-like substances was to study VTG and/or ERα expression and synthesis in male livers; soil pollution by Cd was studied determining Cd content and MT expression and synthesis in different tissues.

12.6 Soil Pollution by Estrogen-like Substances

Under physiological conditions, in *Podarcis* females, during the non-breeding period the plasmatic E2 levels are low and the liver does not synthesize VTG and expresses only the β form of the estrogen receptor. During the breeding period, sexually active females show high levels of circulating E2, in the liver a large amount of VTG is synthesized and ERα and ERβ are co-expressed. In males the VTG gene is physiologically silent and in the liver only ERβ is expressed.

On the other hand, in this lizard the presence of VTG in the liver and plasma of males, in non-breeding females or in sexually immature specimens is considered a hallmark of environmental pollution from xeno-estrogenic substances. In addition, it has been demonstrated that in this lizard the VTG synthesis is also coupled with the expression of ERα in the livers of EDC-exposed samples, so in males even hepatic ERα expression and synthesis may be considered biomarkers of xeno-estrogenic pollution (Figure 12.2).[17]

The first study that we performed to assess if *Podarcis* could be a good sentinel of environmental xeno-estrogenic pollution was aimed at verifying

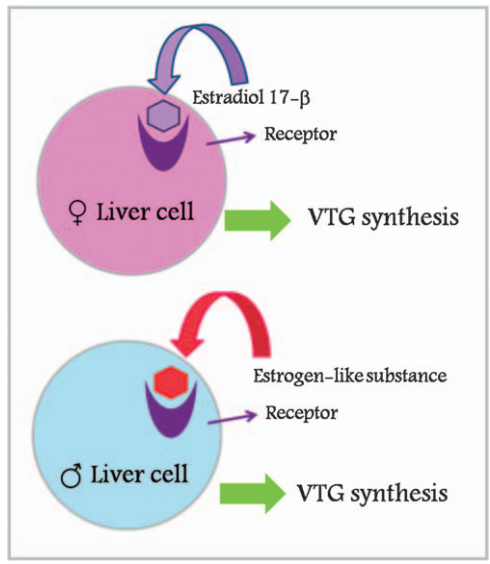

Figure 12.2 Schematic illustration of VTG synthesis, physiologically in females and E2-induced in males.

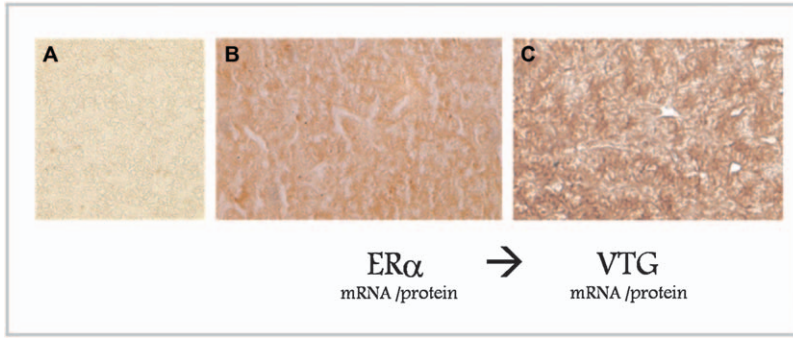

Figure 12.3 In males caught in the outskirts of Naples, in an uncultivated site, no ERα nor VTG was detected (one for all is depicted in (A)). In males caught in areas devoted to organic farming or those fed with NP-polluted food, ERα (B) and VTG (C) are expressed.

the ability of males to synthesize VTG if injected with estradiol-17β. This experimental plan allowed us to demonstrate the ability of males to synthesize VTG in the liver then transfer it to the circulating blood; results also clarified some molecular mechanisms induced by estrogen in the liver. Surprisingly, by means of immunological and biomolecular approaches, we found that, under natural conditions, the only type of estrogen receptor expressed in male livers was ERβ, whereas the treatment with estradiol-17β elicited the transcription and the translation of the ERα. So, it is possible to line up a cascade of events that occur in *Podarcis* liver: estradiol-17β→ ERα→VTG (Figure 12.3).

To establish the effects of an environmental pollution by xeno-estrogen on *P. sicula*, we set up experiments that mimicked the potential conditions to which these terrestrial vertebrates may be exposed. We kept adult male lizards in a terrarium at natural temperature and photoperiod and fed *ad libitum* with food and water contaminated by NP. The results of immunohystochemical and biomolecular investigations undoubtedly showed that 2 weeks of this environmental-like exposure to NP was able to elicit the same cascade of events in the liver: NP→ERα→VTG (Figure 12.3).[59]

The possible expression and synthesis of ERα and VTG was also investigated in livers of wild *Podarcis* males caught in organic farms, where fields are fertilized exclusively by using the manure produced from the animals bred in the same farms. Interestingly, these males also showed appreciable amounts of both ERα and VTG in the liver. No ERα and VTG transcripts and proteins were found in sexually mature males captured in rural, suburban and urban uncultivated areas (Figure 12.3).[60]

Taken together, these observation demonstrate that E2, EDCs and organic substances rich in estrogen metabolites exert the same effects on the male liver; from these results the importance of *Podarcis* males as a sentinel of the pollution by estrogen-like substances also emerges. Further, data show that, although the use of manure as fertilizer avoids chemical pollutants, it may

lead to an accumulation in soils of natural steroids that may affect reproductive processes. Therefore, organic farms should focus more on crop rotation and encouraging biological cycles and soil biological activity rather than using manure as a fertilizer; it is also important to monitor the amount of estrogen derivatives in manure to avoid possible unwanted effects on wildlife and human health. Indeed, in recent years, it has been estimated that organic farming has grown by 8.9% per year;[61] so, if nothing is done to mitigate the problem, soil contamination by steroids and EDC could soon represent a risk as serious as soil contamination by chemicals and heavy metals is currently.

At this point, it is easy to assume that exposure to EDCs may interfere with the correct progress of the reproductive cycle. Hence, we demonstrated that in *P. sicula* males treated with estradiol-17β, spermatogonia acquire the ability to synthesize VTG.[60] Now, we cannot say whether these germ cells synthesizing VTG are reproductively efficient or are damaged. However, morphological analysis demonstrated that the E2 treatment impaired the *P. sicula* spermatogenesis, in a time-dependent manner. After 2 weeks of treatment, the spermatogenesis is slowed, as evident from the wide lumen of the seminiferous tubules and the several empty spaces among germ cells. The major alterations are observed after 8 weeks of treatment, with the seminiferous epithelium surrounded by abundant connective tissue. In addition, we also observed the presence inside the tubules of some oocyte-like structures, which are reported as typical alterations of testis germ cells exposed to estrogens.[62–67] Both estrogenic treatments also affected the epididymis, which appeared regressed, with no sperms and fluids in the lumen of the ducts, resembling the typical condition of the epididymis during the non reproductive period.[60]

Similar results were observed in lizards exposed to an NP-polluted diet or collected in manure-treated areas. Hence, in these two conditions, VTG is expressed in the testis in addition to the liver; however, many differences were observed at the histological level in the three different groups of animals. Impairment of spermatogenesis and alterations in testicular and epididymal structures have been observed only in *Podarcis* males fed with larvae polluted by nonylphenol, whereas in males from the organic farming areas the testis and epididymis appeared morphologically normal, in accordance with the reproductive period in which the animals were collected.[68]

The response of *Podarcis* females to estrogen exposure is much less deleterious than that for males. In fact, E2 treatment during the breeding period causes early reproductive cycle closure, whereas the ovary condition both in NP polluted samples or in animals collected in BIO areas is consistent with the breeding season (our unpublished data).

12.7 Soil Pollution by Pesticides

Ecotoxicological studies carried out in *Podarcis* with three different pesticides, *i.e.* imidacloprid, methyl thiophanate or diuron, showed severe damage at the reproductive level.

In particular, recent investigations showed that the exposure to imidacloprid, a neonicotinoid insecticide acting as an agonist of nicotinic acetylcholine receptors, affects spermatogenesis in *P. sicula* males. The study demonstrated that the spermatogenesis stopped, with a concomitant increase of germ cell apoptosis and a decrease of steroid receptor mRNAs.[69]

Similar results were achieved with the exposure of these lizards to the fungicide methyl thiophanate (Mt). Intraperitoneal injections of Mt in male specimens led to a reduction of the testicular lumen and the number of the germ cells with an increase in germ cell apoptosis and a decrease in the expression of androgen and estrogen receptor mRNAs.[70]

Finally, the exposure to the herbicide diuron caused in *Podarcis* hypertrophy of the interstitial connective tissue of the testis and a slowdown of spermatogenesis, probably owing to an induction of the necrosis process. Furthermore, the epididymal structures regressed and the level of sexual steroids was negatively affected.[71]

12.8 Soil Pollution by Cadmium

Determination of cadmium content in the liver of wild *P. sicula* specimens demonstrated that Cd accumulation was correlated with different sampling sites (Figure 12.4). Cd levels were significantly higher (about fivefold) in animals collected in uncultivated areas in the outskirts of Naples with respect to animals collected in fields devoted to organic farming.[60]

To investigate the influence of dietary Cd exposure on the tissue distribution and accumulation of Cd ions in terrestrial vertebrates, some lizards were caught, kept in a terrarium under natural conditions of temperature and photoperiod, and fed every second day for 60 days with 1 μg $CdCl_2$ per g

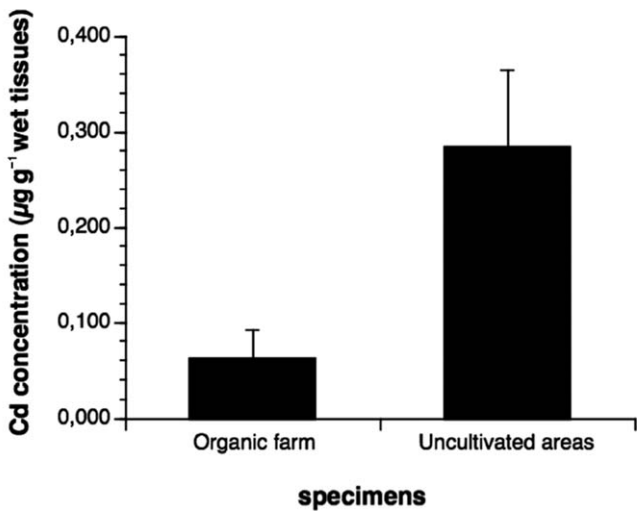

Figure 12.4 Cadmium content in *P. sicula* male livers. Values are expressed as mean ± S.D. ($n = 15$).

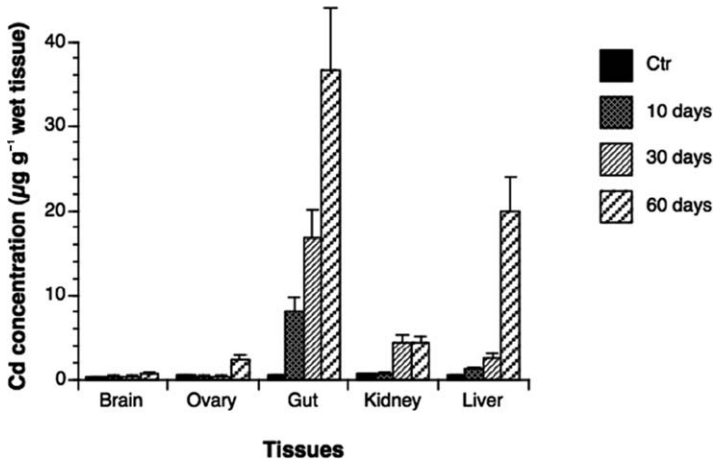

Figure 12.5 Cadmium accumulation in lizard tissues during dietary cadmium treatment (1 μg Cd g^{-1} bodyweight *via* gavage) every second day for 60 days. Values are expressed as mean ± S.D. ($n = 10$).

of body weight *via* gavage.[70] Cadmium accumulation in the liver, kidneys, ovaries, brain and intestine was measured by atomic absorption spectrometry at 10, 30 and 60 days of treatment (Figure 12.5).[70]

As expected, the first site of orally administered Cd accumulation in *P. sicula* was the gut; then, probably *via* passive diffusion or H(+)-antiport as in mammalian cells, Cd was secreted by intestinal cells reaching other tissues. At the end of treatment, we found that the main target organs were the gut and liver, followed by the kidneys, ovaries and brain. In the last two organs, only prolonged treatment of 60 days gives rise to a significant Cd accumulation.[46,70]

A large body of evidence demonstrates that in fish and mammals exposed to chronic Cd intoxication, the kidney is the main target organ. Our findings demonstrated the importance of using multiple model organisms in biomonitoring studies.[72–74]

Together with cadmium content, we also investigated MT mRNA (Figure 12.6) accumulation after chronic Cd treatment. Under the experimental conditions described, a relationship between Cd accumulation and MT transcript induction is not always possible to establish, but it appears to be dependent on the organ.

When lizards were exposed to dietary Cd, the amount of MT transcript dramatically increased in the gut after 30 days of treatment, whilst in the ovary and kidney the increase was detectable only after 60 days of treatment; no MT induction at the RNA level was detectable in the brain and liver throughout the treatment.[70] Hence, data suggest that, although MT mRNA induction in the gut can be used as a clear indicator of dietary Cd intoxication, in other lizard tissues the MT mRNA level is not a suitable tool for the detection of low levels of Cd contamination in the environment.

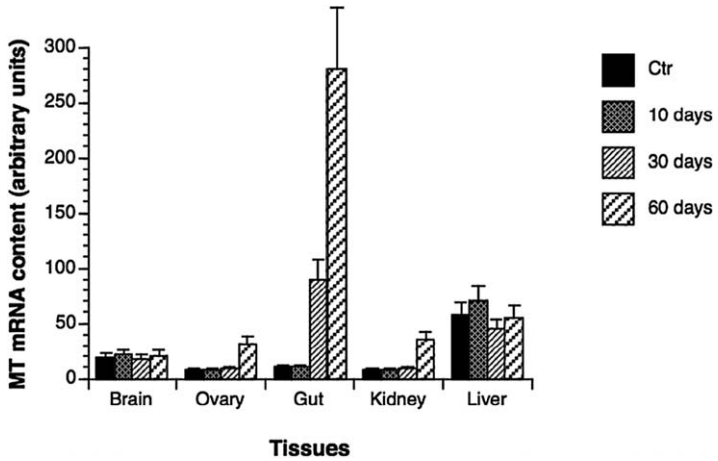

Figure 12.6 Metallothionein mRNA expression in *P. sicula* organs during chronic cadmium treatment. Dot blots of total RNA (5 μg) from tissues of control and Cd-treated animals were probed with a cDNA fragment corresponding to the *P. sicula* MT coding sequence. The histogram shows the amounts of MT mRNA estimated using Image Quant Software (Molecular Dynamics). Each bar is the average of measurements carried out on three distinct blots.

12.9 Conclusions

In conclusion, data herein illustrated demonstrate that the lizard *Podarcis sicula* is a good sentinel of environmental wellness. In particular, the availability and wide spread in nature, together with the full knowledge of its life cycle, make this terrestrial vertebrate an excellent animal model to study the potential toxic effects of emerging terrestrial pollutants, both of natural and synthetic origin, and almost a unique model for investigating the welfare of wild terrestrial vertebrates in field studies.

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CHAPTER 13

The Yellow-legged Gull Larus michahellis *(Charadriiformes, Laridae) as a Model Species in Ecotoxicology: Application in Monitoring and Toxicity Assessment of Environmental Pollutants*

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13.1 Introduction

For a long time, ecotoxicology has focused on the risks associated with the presence of persistent organic pollutants (POPs) in terrestrial ecosystems. POPs are persistent and widespread xenobiotics. Because of their fat solubility and resistance to both chemical and biological degradation, POPs can be accumulated in fat tissues of living organisms, increasing their concentration at high trophic levels, and may exert diverse toxic effects at different

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levels of the biological organization.[1] Although most POPs are currently banned and are no longer produced or used in most parts of the world, resulting in constantly declining environmental levels,[2–4] considerable amounts of them still persist in both abiotic and biotic matrices. In recent years, the issue concerning the chemical contamination of natural ecosystems has received even more attention because of growing evidence of the presence of so-called emerging pollutants in several environmental matrices. These xenobiotics are new products or chemicals with no regulatory status, whose environmental and health effects are unknown.[5] Among them, perfluoroalkyl and polyfluoroalkyl substances (PFASs) have aroused concern because of their widespread distribution in the environment and scarce information on their potentially adverse effects on living organisms. PFASs are used in diverse industrial and consumer products, mainly to repel dirt, water and oil.[6] Overall, they are non-volatile molecules resistant to physical and biological degradation, which impart many of them with high environmental stability.[7,8] Perfluorooctane sulfonate (PFOS; $C_8F_{17}SO_3^-$) is a PFAS that has long been used in industrial and commercial applications.[8–10] Although its use is currently regulated,[11] because of its peculiar chemical–physical features and bio-accumulative nature, PFOS is commonly found in both aquatic and terrestrial environments, mainly in tissues of top-predator species.[12–14] Although monitoring studies have revealed the presence of POPs and emerging pollutants, including PFASs, in aquatic ecosystems, there is a dearth of information regarding their occurrence in terrestrial ecosystems. In addition, the information on the adverse effects induced by exposure to these xenobiotics in both aquatic and terrestrial organisms is largely inadequate.

Bird species have been commonly used as sentinels to monitor the levels of POPs in the terrestrial environment, because they are exposed to natural and anthropogenic pollutants that can accumulate through trophic transfer and direct uptake.[15] Monitoring studies generally rely on common top-consumers that are exposed to relatively high concentrations of diverse environmental pollutants, which can be accumulated in their soft tissues at high concentrations *via* biomagnification, reflecting the contaminant burden of the female at the time of laying and integrating the pollutant levels over a large area.[16,17] Because the eggs incorporate maternal lipophilic xenobiotics[18,19] they have been proposed as a non-invasive tool to assess the levels and trends of POPs in terrestrial ecosystems (Stockholm Convention, United Nations Environmental Programme (UNEP) and the Oslo Paris Convention (OSPAR)).[20] Several studies have reported high levels of POPs, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (*e.g.*, p,p′-DDT and its homologues, hexachlorocyclohexane isomers), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), and flame retardants in eggs of several bird species.[21–24] In addition, recent monitoring investigations have shown the presence of emerging contaminants, including different PFASs, in the eggs of the common shag (*Phalacrocorax aristotelis*),[25] cormorant (*Phalacrocorax carbo*),[26] little egret (*Egretta garzetta*)[27,28] great blue heron (*Ardea herodias*) and

tree swallow (*Tachycineta bicolor*)[29–31] in the 0.29–9453 ng g^{-1} wet weight range, confirming that these chemicals can be considered terrestrial contaminants that are maternally transferred to eggs.[20]

These findings reinforce the necessity to shed light on the distribution of both legacy and emerging pollutants in the terrestrial environment and, above all, to investigate their potential toxicity towards terrestrial wild species. In fact, many studies have shown that bird egg xenobiotics accumulated in bird eggs can affect embryo development, offspring health status and individual fitness.[32,33] These adverse effects are particularly clear in the embryo because early developmental stages have been recognized as the most sensitive period in the organism's life cycle to contaminant exposure.[34] For instance, diverse malformations have been found in guillemot (*Uria aalge*) embryos from the Baltic Sea and Atlantic Ocean, possibly as a result of egg concentrations of some POPs.[35] However, in spite of these correlative findings, identifying the causal relationships between individual chemicals and the observed adverse effects may be problematic because of the positive covariation of the concentrations among pollutants and their interaction effects. Thus, the toxicity of maternally derived compounds can be studied by two different experimental approaches. The first one considers the supplementation of contaminated food to mothers, but it may not be totally satisfactory because it does not disentangle the indirect effect of the focal chemical as mediated by its consequences for maternal physiology and egg quality, from its direct effect. The second one investigates the toxicity of maternally transferred chemicals through the experimental manipulation of their concentration into the egg, avoiding confounding effects mediated by maternal physiology.[36,37] Recent studies of avian species have focused on sub-lethal effects caused by some classes of emerging environmental pollutants, mainly polybrominated diphenyl ethers (PBDEs), showing adverse effects on egg hatchability, embryo development, hatchling and adult phenotype, as well as reproduction and fitness.[36,38,39] However, only a few studies have investigated PFOS toxicity in birds. For instance, *in ovo* PFOS supplementation reduced egg hatchability and caused hepatic aberrations in white leghorn chickens (*Gallus gallus*),[40] while acute and chronic exposure resulted in reduction in egg hatchability and hatchling survival, decrease of weight gain and hepatomegaly in mallards (*Anas platyrhynchos*) and Northern bobwhites (*Colinus virginianus*).[41,42] These studies were performed under controlled laboratory conditions on conventional biological models of ecotoxicology, testing concentrations much higher than those currently found in eggs from free-living species, and focusing on a limited number of endpoints. For example, they completely neglected the occurrence of oxidative stress in PFOS-treated individuals, which has been suspected as one of the main causes of developmental toxicity in embryos of vertebrate species.[43–45]

The present chapter presents an innovative experimental approach on a non-conventional model organism for ecotoxicology to investigate the toxicity of an emerging environmental pollutant. Specifically, we demonstrate

Figure 13.1 The yellow-legged gull (*Larus michahellis* – (A)) and its eggs in a typical nest (B).

the applicability and the reliability of an *in ovo* supplementation method to assess adverse effects of PFOS on embryos at the cracking stage of a free-living species, the yellow-legged gull (*Larus michahellis*), under a natural selection regime.

The yellow-legged gull (Figure 13.1) is a monogamous species inhabiting mostly coastal habitats across the Mediterranean, where it often breeds colonially.[46] Clutch size ranges between one and three eggs, whichare laid at 1–4 (most frequently 2) day intervals and hatch 27–31 days after laying. Hatching is asynchronous, spanning over 1–4 days. The chicks are nidifugous and altricial, are fed by both parents and fledge at 35–40 days of age.[46] The yellow-legged gull can be considered an excellent non-conventional biological model for terrestrial ecotoxicological studies because:

(1) It has an extremely large geographical distribution, positive population trends and very large population size. Thus, the species is evaluated as Least Concern.[47]
(2) It is a widespread species that can be found in Europe, the Middle East and North Africa. It is resident in much of southern Europe, on the coasts of the Mediterranean, Black Sea and Caspian Sea, on the Azores and Madeira, Portugal, and on the Canary Islands.[47]
(3) It is an omnivorous species and its diet consists of fish, invertebrates (including insects, mollusks and crabs), reptiles, small mammals, offal, and bird eggs and chicks.[47] In addition, it also scavenges on rubbish. Its feeding behavior entails the accumulation of diverse lipophilic compounds, which can then be maternally transferred to the eggs.
(4) It lays eggs on the ground and the nest is easily accessible.
(5) Eggs are big (>70 g) and can be easily experimentally manipulated. In addition, yolk (>15 g fresh weight *per* egg) is rich in lipids and can 'store' a great amount of contaminants, which renders them a good bioindicator of environmental pollution.[48]
(6) Considering its conservation status and widespread distribution, the collection of eggs has negligible effects on the population dynamics of the species.

For all the above reasons, the yellow-legged gull is a good candidate species to investigate the presence of environmental pollutants in focal geographical areas, the processes regulating the maternal transfer of toxic molecules,[49] as well as their effects on embryos and hatchlings.

In the present study, we manipulated PFOS concentrations in yellow-legged gull eggs through *in ovo* injections and we assessed PFOS-induced effects on: (1) morphometric endpoints, namely body mass, liver and brain mass and tarsus length of developing embryos, (2) embryo oxidative status, by measuring the Total Antioxidant Capacity (TAC) and the Total Oxidant Status (TOS) in the liver and brain, and (3) oxidative and genetic damage in both the target organs.

13.2 Materials and Methods

13.2.1 Study Area

We studied a large colony (>400 pairs) of yellow-legged gull settled on an island in the Comacchio lagoon (NE Italy, 44°20′ N – 12°11′ E) in March–May 2014. At the beginning of the breeding season, we visited the study colony every second day. New nests were marked with a labeled stick, and the newly laid eggs were labelled with a waterproof marker. When a new egg was found, it was temporarily removed from the nest and taken to a nearby tent for manipulation, and temporarily replaced with a 'dummy' egg to avoid interference with the incubation behavior of the parents.

13.2.2 *In Ovo* PFOS Manipulation

We adopted a within-clutch experimental design, with both control and PFOS-injected eggs within each clutch, to minimize the consequences of environmental and parental effects.[50] Eggs were injected in the albumen. Before being injected, each egg was placed with the longitudinal axis vertical in a safe position for 15 min. Then, the eggshell close to the acute pole was disinfected and a hole was drilled by means of a sterile pin. Injections were made using a 1 mL sterile syringe mounting a 0.6×30 mm needle. We injected PFOS in egg albumen rather than in yolk because of its amphipathic properties and its high binding affinity to proteins, such as lipoproteins and albumin.[51] In addition, since the albumen is used in early developmental stages while yolk is only used for late body growth of the embryo, we can assume that the embryo is exposed to all the injected amount of PFOS. We injected the eggs with two environmentally relevant PFOS concentrations, namely 100 ng PFOS g^{-1} egg weight and 200 ng PFOS g^{-1} egg weight, which were respectively two- and four-fold higher than the maximum PFOS level measured in yellow-legged gull eggs from the Iberian Peninsula (range 10.1–54.0 ng PFOS g^{-1} wet weight).[20] In addition, the lowest tested concentration corresponds to the lowest observed adverse effect level (LOAEL) on hatchability after PFOS *in ovo* injection in leghorn chicken.[40] We injected

30 μL of PFOS solutions, while control eggs were injected with 30 μL of dimethyl sulfoxide (DMSO). We used DMSO as the carrier solvent because it is not embryotoxic.[52] Immediately after injection, a small piece of eggshell was superimposed onto the hole, which was sealed with a drop of epoxidic glue. Injected eggs were taken back to their original nest within 3 hours and the dummy eggs were removed. The clutches were assigned sequentially to the following treatment schemes (nest, first-, second- and third-laid egg), according to the order in which the first egg was found: nest 1, 100 ng PFOS g^{-1} egg weight (D1), control (C), 200 ng PFOS g^{-1} egg weight (D2); nest 2, C-D1-D2; nest 3, D2-D1-C; nest 4, D2-C-D1 and so forth with the following nests. For simplicity, we named the first-, second- and third-laid eggs as a-egg, b-egg and c-egg, respectively. We injected eggs in 53 nests. Since there is no evidence regarding the variation of PFOS concentration in yellow-legged gull eggs according to the egg size and/or the position in the laying order, we did not modulate the PFOS concentrations to be injected according to these variables. All the nests were visited every day starting 5 days before the earliest expected hatching date to check for any sign of imminent hatching (eggshell cracks). When the eggshell was cracked (cracking stage), the egg was collected and frozen at −20 °C until the dissection of the embryo. The embryos were weighed (to the nearest g) and their tarsi were measured by calipers (to the nearest mm). Then, embryo liver and brain were dissected, weighed (to the nearest mg) and frozen at −80 °C until biochemical analyses. All the measurements were taken by a single person to ensure consistency. Molecular sexing of each single embryo was performed on a small piece of liver according to a previously validated method.[53]

13.2.3 PFOS Determination in Yolk Sac from Control Eggs

To verify the ecological relevance of injected PFOS concentrations in our study, we measured the concentration of PFOS in the residual yolk sac from control egg embryos. Since the residual yolk in yellow-legged gull eggs at the cracking stage was higher than 70% of its total amount at the time of deposition (personal observation), the analysis of PFOS levels in the yolk likely gives a reliable indication about the maternally transferred PFOS concentrations. PFOS levels were measured in control eggs from 21 three-egg clutches ($n = 6$ a-eggs, $n = 9$ b-eggs and $n = 6$ c-eggs).

The extraction of PFOS from yolk samples was carried out according to Lacina *et al.*,[54] with slight modifications. Briefly, about 1 g of fresh yolk was spiked with 100 μL of a 13C4-PFOS solution (40 μg L^{-1} in methanol). 5 mL of water–acetonitrile solution (10 : 90 v/v) and 70 μL of concentrated formic acid (≥98%) were added to the sample and vigorously shaken. The mixture was sonicated for 10 min, centrifuged at 11 000 rpm for 10 min at 10 °C and the supernatant transferred to a clean tube. The extraction was performed in triplicate. Then, 0.5 g of NaCl and 2 g of $MgSO_4$ were added to the supernatants. After vigorous shaking, the mixture was centrifuged and stored at −21 °C overnight. The supernatant was reduced under a gentle nitrogen

stream to 1 mL, acidified with 50 μL of formic acid and filtered through HybridSPE® Phospholipid Ultra cartridge (Supelco, 30 mg, 1 mL SPE Tubes) to remove phospholipids.[55] PFOS concentration was determined by liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) coupled to turbulent flow chromatography (TFC). The extract was cleaned up online through two serially connected TFC columns (Thermo Fluoro XL, 50×0.5 mm and Thermo Cyclone™, 50×0.5 mm) using a modified CTC PAL autosampler equipped with three six-way VICI valves and two Thermo Scientific Accela (600 and 1200) LC pumps. The extract (50 μL) was transferred onto the TFC columns by a loading pump (Thermo Scientific Accela 600) using 1% HCOOH eluent at 2000 $\mu L\,min^{-1}$ to promote the discharge of proteins and salts. The trapped analytes were eluted from the TFC columns into the analytical stream by a plug of 100 μL of MeOH for the subsequent analysis. UHPLC-MS/MS analysis of PFOS was carried out according to Mazzoni *et al.*[56] Analytes were separated using a Hypersil GOLD PFP (1.9 μm, 50×2.1 mm, Thermo) column and a chromatographic gradient of 2 mM ammonium acetate (5% methanol) and methanol at 300 $\mu L\,min^{-1}$. A QqQ mass spectrometer (Thermo Scientific TSQ Quantum Access MAX) equipped with a heated electrospray ionisation (HESI-II) probe operating in negative mode was used. Quantification was done using the isotopic dilution method.

13.2.4 Oxidative and Genetic Biomarker Methods

All biomarker analyses were performed on liver and brain dissected from yellow-legged gull embryos and described in detail elsewhere.[50] Briefly, organs were homogenized in a buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton-X and 1× protease inhibitors) by an automatic homogenizer, and the protein content of the homogenates was immediately determined according to the bicinchoninic acid method (BCA). Total Antioxidant Capacity (TAC) was measured as the reduction of a phosphomolybdate complex by the antioxidants in the sample.[57] When the complex is reduced, it forms a dark-blue chromophore that can be measured spectrophotometrically at $\lambda = 750$ nm. The method was calibrated by using ascorbic acid and the TAC level was expressed as $\mu g\ mg^{-1}$ protein. TOS was measured through a colorimetric method according to which the oxidants in the sample oxidize the ferrous ion-*o*-dianisidine complex to the ferric ion, which reacts with xylenol orange to give a blue complex.[58] The assay was calibrated by using hydrogen peroxide (H_2O_2) and the results are expressed as nM H_2O_2 equivalent g^{-1} wet weight. The oxidative status index (OSI) was calculated as the TOS/TAC ratio for each specimen; high ratios reflected a marked imbalance of the oxidative status. The TAC assay had an average intra-assay coefficient of variation (CV) of 7.4% ($n = 3$ replicates) and the inter-assay CV was 13.5% ($n = 3$ assay plates). For TOS the average intra-assay CV was 6.0% ($n = 3$ replicates) and the inter-assay CV was 12.6 % ($n = 3$ assay plates).

Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH). Derivatized proteins were detected by Western immunoblotting and

immunostained protein bands were visualized with enhanced chemiluminescence detection. Carbonylated proteins were quantified by densitometric analysis using Image J 1.40d software. A single assay for each sample was performed, so no intra- or inter-assay variation can be calculated.

DNA strand breaks were quantified in duplicate by using a fluorescence technique adapted from the alkaline precipitation assay.[59] Raw homogenate samples were mixed with 2% SDS containing EDTA (10 mM), tris-base (10 mM) and NaOH (40 mM). After KCl (0.12 M) addition, the solution was heated at 60 °C for 10 min, mixed by inversion and cooled at 4 °C for 30 min. Then, the solution was centrifuged at 13 000 rpm for 5 min at 4 °C and an appropriate volume of supernatant was added to Hoescht 33258 dye solution (1 $\mu g\,mL^{-1}$ in 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-acetate buffer, pH 8.5–9). The fluorescence of the samples was measured using 360 nm (excitation) and 450 nm (emission) filters against a blank. Assay calibration was performed by using salmon sperm genomic DNA standards and the results are expressed as μg DNA mg protein^{-1}.

13.2.5 Statistical Analysis

The analysis of embryo viability until the egg cracking stage (*i.e.* 'cracking success') was performed on the entire sample of injected eggs ($n = 159$ eggs from 53 clutches), whereas for the analyses of morphological and biochemical endpoints we considered only clutches where three embryos reached the cracking stage ($n = 63$ eggs from 21 clutches). The effect of PFOS treatment on embryo morphological and biochemical variables was analyzed by means of linear mixed models (LMM), including PFOS treatment, embryo sex and laying order as fixed factors together with their two-way interactions. Egg mass at laying was included as a covariate, while clutch identity was entered as a random intercept effect. Interaction terms were retained in the models even when statistically non-significant. LMM with the same design while assuming a binomial error distribution were run to assess the effect of PFOS treatment on the proportion of eggs that reached the cracking stage and on the embryo sex ratio at the cracking stage. The statistical analyses were run using SAS 9.3 PROC MIXED and PROC GLIMMIX.

13.3 Results and Discussion

This study was aimed at investigating the developmental and oxidative alterations following manipulation of PFOS levels in embryos of yellow-legged gulls under natural selection regime.

13.3.1 PFOS Concentrations in Control Eggs

Concentrations of PFOS ranging between 31 and 687 $ng\,g^{-1}$ wet weight (mean 166 ± 37 $ng\,g^{-1}$ wet weight) were found in the residual yolk sac of embryos from control eggs. The highest PFOS concentration measured in the present study was about 13-fold higher than the maximum value found

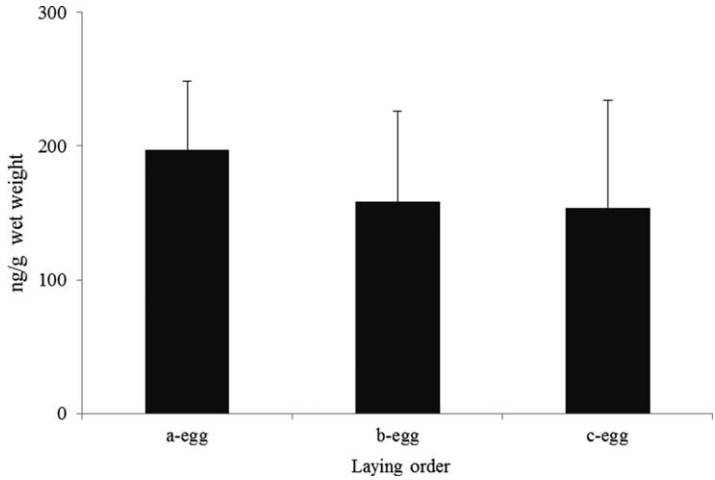

Figure 13.2 Mean concentration (±SE) of PFOS (ng PFOS g^{-1} wet weight) in residual yolk sacs from control eggs ($n = 6$ a-eggs, $n = 9$ b-eggs and $n = 6$ c-eggs). (Reproduced with permission from ref. 50).

in yellow-legged gull eggs from the Iberian Peninsula (range 10–54 ng g^{-1} wet weight).[20] PFOS concentrations did not vary according to the laying order ($F_{2,18} = 0.106$; $P = 0.900$; Figure 13.2), differently from results in Audouin's gull (*Larus audouinii*) eggs.[60]

13.3.2 PFOS Effects on Embryo Development and Morphometric Traits

The results of the chemical analyses confirmed that the injection of 100 ng PFOS g^{-1} and 200 ng PFOS g^{-1} egg weight realistically mimics the PFOS environmental levels of yellow-legged gull eggs in the study population. *In ovo* injection of PFOS concentrations did not affect cracking success, as gauged by the proportion of eggs that reached the cracking stage, that did not significantly differ ($F_{2,105} = 0.79$; $P = 0.924$) between control (42/53 = 0.79) and PFOS-injected eggs (D1 = 41/53 = 0.77; D2 = 39/53 = 0.73). These results are consistent with previous findings from manipulation of PFOS concentrations (0.1 and 5 μg PFOS g^{-1} egg) in white leghorn eggs, showing no significant increase of embryo mortality.[52,61] However, a previous study of the same species showed a LOAEL based on embryo mortality results of 100 ng PFOS g^{-1} egg.[40] The discrepancy between results of the two *in ovo* manipulation studies on chickens could depend on the orientation in which eggs were placed in the incubator, as noted in a previous study investigating the embryotoxicity of methyl mercury (MeHg) injected in eggs of chickens, mallards and ring-necked pheasants.[62] In fact, Molina *et al.*[40] incubated eggs in the vertical position, while O'Brien *et al.*[52] incubated them horizontally, mimicking the natural situation. These findings suggest that the orientation of the egg during incubation may overestimate the toxicity

of injected chemicals, implying that our choice to allow a natural parental incubation provided a correct evaluation of PFOS toxicity.

PFOS injections did not significantly affect embryo body mass ($F_{2,33.7} = 0.753$, $P = 0.478$) and tarsus length ($F_{2,37} = 2.974$, $P = 0.063$) (Figure 13.3A and B, respectively) after controlling for the potentially confounding effects of sex and laying order,[50] and agree with a previous study of white leghorn chicks.[61] The same study also investigated variation in brain and liver mass, revealing that concentrations much higher than those we established in yellow-legged gull eggs did not cause brain mass reduction, but affected brain symmetry. No significant change in brain ($F_{2,36} = 0.061$, $P = 0.941$; Figure 13.3C) and liver ($F_{2,37.2} = 0.615$, $P = 0.546$; Figure 13.3D) mass was induced by PFOS treatment compared to controls. Our results disagree with those from previous studies indicating that *in ovo* PFOS supplementation caused duct hyperplasia, periportal inflammation and cellular necrosis in embryos of white leghorn chickens,[40] while significant hepatomegaly was noticed in mallard (*Anas platyrhynchos*) and Northern bobwhite (*Colinus virginianus*) specimens after dietary exposure to PFOS.[42] In addition, a field study of great tits (*Parus major*) and blue tits (*Parus caeruleus*) showed a significant correlation between the increase in alanine aminotransferase activity and the decrease in serum cholesterol and triglyceride levels, and increasing PFOS concentrations in blood, suggesting that this chemical may affect lipid metabolism.[13] Despite these findings, the lipid content of the liver from the yellow-legged gull embryos was not significantly affected by PFOS exposure.

13.3.3 PFOS Effect on Oxidative Stress and Genetic Biomarkers

Previous studies showed that exposure to different PFASs, including PFOS, can induce the overproduction of reactive oxygen species (ROS), promoting oxidative damage *via* peroxisome proliferator-activated receptor (PPAR) activation.[63] Accordingly, our *in ovo* PFOS injection could alter the oxidative status of embryos and cause oxidative damage to cellular macromolecules, such as lipids, proteins and DNA. Even if no investigation has been currently focused on PFOS-induced oxidative damage to bird species this hypothesis was supported by an *in vitro* PFOS exposure of primary hepatocyte cultures from tilapia (*Oreochromis niloticus*), which induced ROS overproduction and unbalances of antioxidant responses, resulting in lipid peroxidation and DNA damage.[64] In addition, *in vivo* PFOS treatment caused oxidative damage in the liver of different fish species,[65] impaired the homeostasis of the antioxidant system and induced an oxidative stress situation in rat offspring.[66] Our results showed that PFOS treatment did not significantly imbalance the oxidative status in the liver of yellow-legged gull embryos. No significant effect of PFOS treatment on liver TOS ($F_{2,25.2} = 0.714$, $P = 0.499$) and TAC ($F_{2,27} = 0.700$, $P = 0.932$) was found (Figure 13.4A and B,

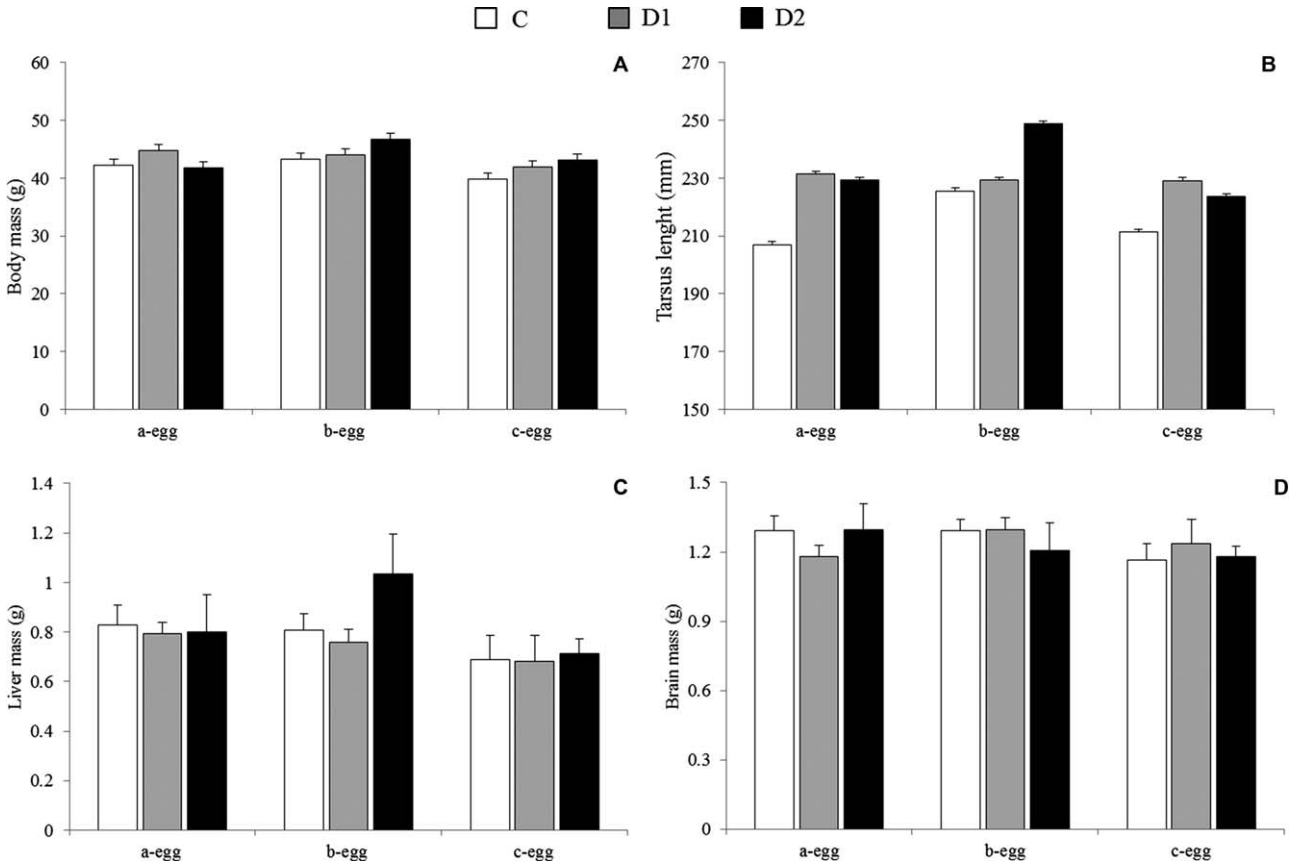

Figure 13.3 Mean (±SE) body mass (A), tarsus length (B), liver (C) and brain mass (D) in PFOS treatment (C = control; D1 = 100 ng PFOS g^{-1} egg; D2 = 200 ng PFOS g^{-1} egg) and laying order groups in yellow-legged gull embryos ($n = 21$ three-egg clutches). No statistically significant effect of treatment, laying order and their interactions was found for each endpoint. (Reproduced with permission from ref. 50).

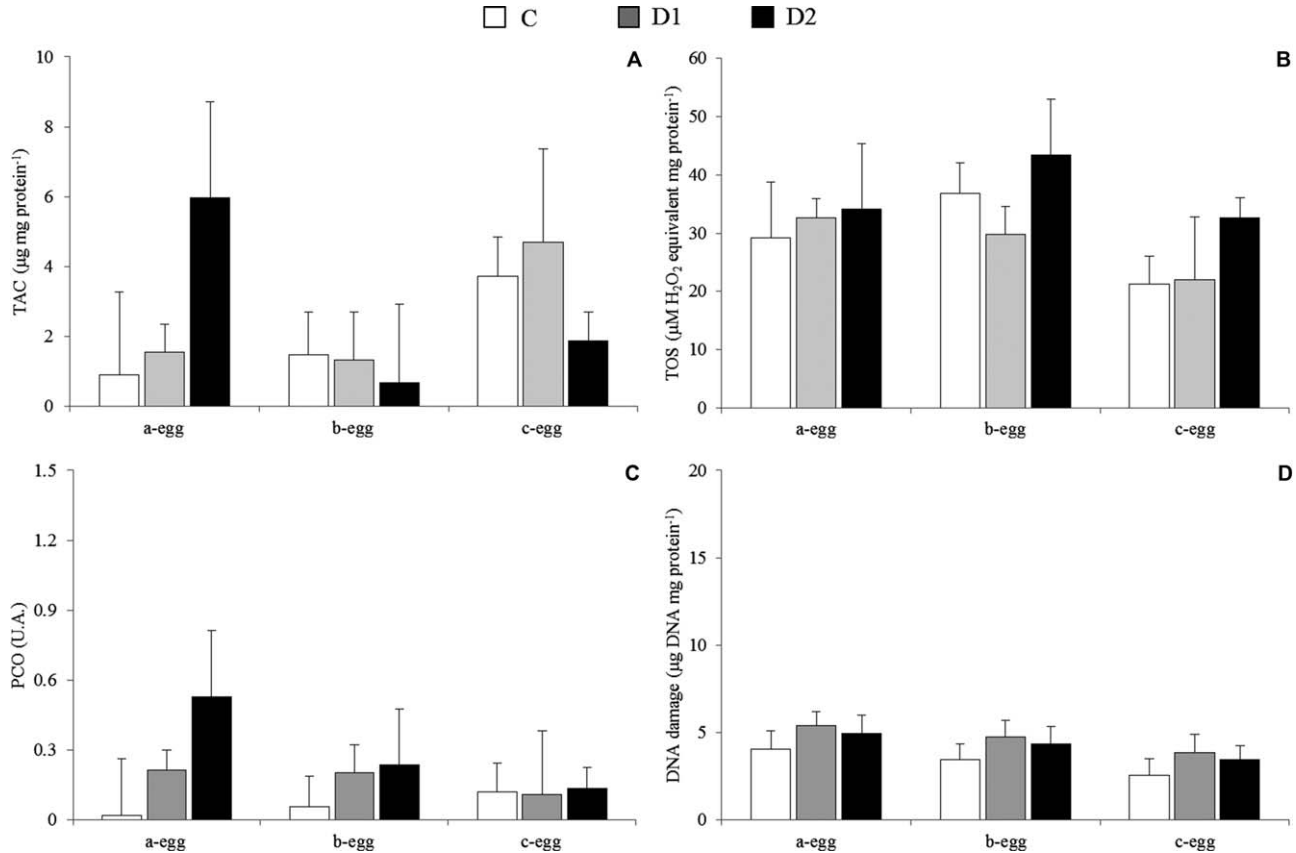

Figure 13.4 Mean (±SE) of Total Antioxidant Capacity (TAC, A), Total Oxidant Status (TOS, B), Protein Carbonyl content (PCO, C) and DNA fragmentation (D) in the PFOS treatment (C = control; D1 = 100 ng PFOS g^{-1} egg; D2 = 200 ng PFOS g^{-1} egg) and laying order groups in the liver from yellow-legged gull embryos ($n = 21$ three-egg clutches). No statistically significant effect of treatment, laying order and their interactions was found for each endpoint.
(Reproduced with permission from ref. 50).

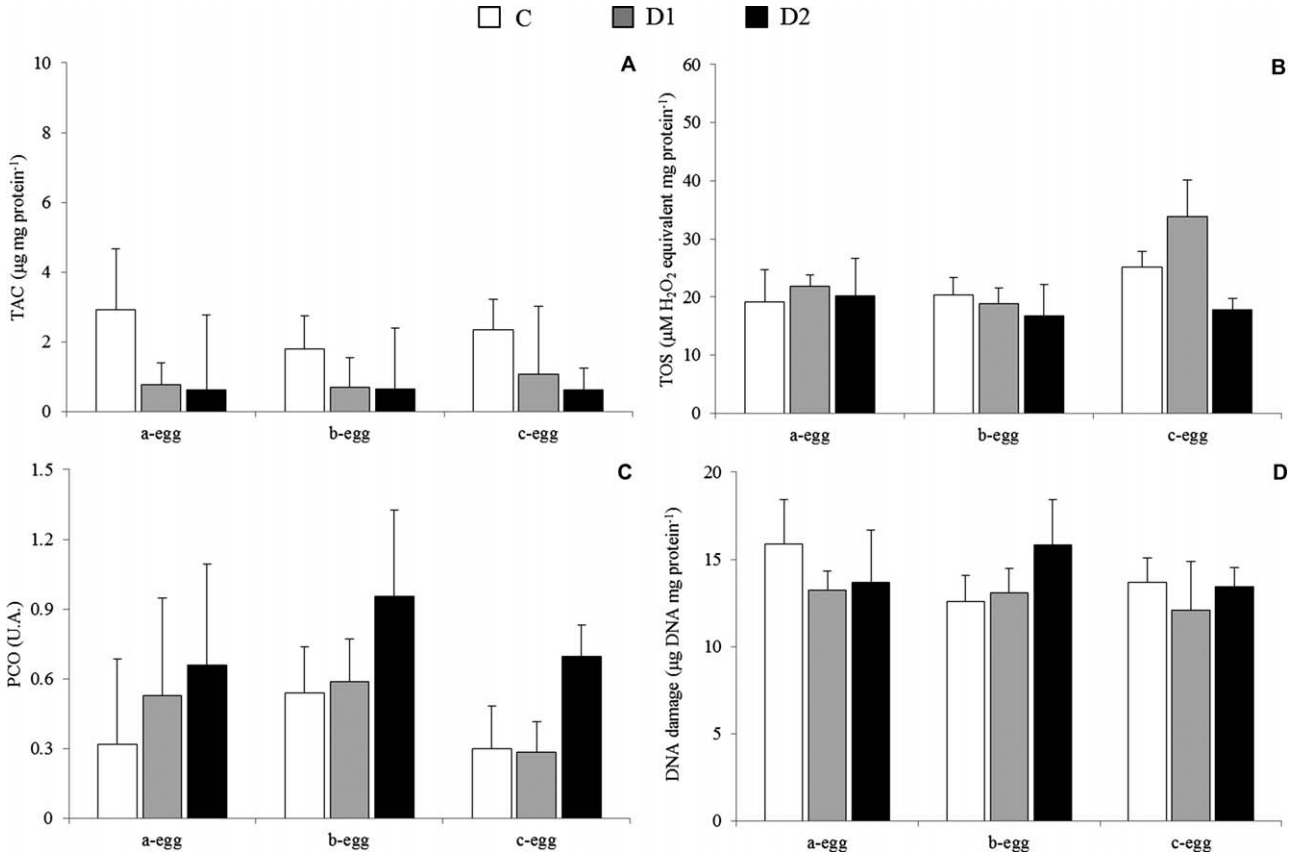

Figure 13.5 Mean (±SE) of Total Antioxidant Capacity (TAC, A), Total Oxidant Status (TOS, B), Protein Carbonyl content (PCO, C) and DNA fragmentation (D) in the PFOS treatment (C = control; D1 = 100 ng PFOS g^{-1} egg; D2 = 200 ng PFOS g^{-1} egg) and laying order groups in the brain from yellow-legged gull embryos ($n = 21$ three-egg complete clutches). No statistically significant effect of treatment, laying order and their interactions was found for each endpoint.
(Reproduced with permission from ref. 50).

respectively). The analysis of OSI, calculated as the TOS/TAC ratio, confirmed that injected PFOS concentrations did not disrupt the equilibrium between pro-oxidants and antioxidants in treated embryos with respect to controls. Accordingly, no significant effect of PFOS on liver oxidative damage, assessed by levels of protein carbonylation ($F_{2,24.2} = 0.616$, $P = 0.548$), was found (Figure 13.4C).

Similarly, no effects of PFOS injection on TOS ($F_{2,28} = 1.810$, $P = 0.182$), TAC ($F_{2,23.4} = 1.137$, $P = 0.338$) and protein carbonyl content ($F_{2,28} = 0.660$, $P = 0.525$) were noticed in homogenates of brain from treated specimens compared to controls (Figure 13.5A–C). Lastly, even if PFOS treatment induced primary DNA lesions and promoted the apoptotic process in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*),[64] no significant increase of DNA fragmentation was caused by PFOS injection in both liver ($F_{2,28} = 0.901$, $P = 0.417$) and brain ($F_{2,26.8} = 0.822$, $P = 0.432$) dissected from treated yellow-legged gull embryos compared to controls (Figures 13.4D and 5D, respectively). Overall, our data should suggest that tested PFOS concentrations might be considered as non-genotoxic for yellow-legged gull embryos.

13.4 Conclusions

The present study showed that the *in ovo* injection of two PFOS at environmentally relevant concentrations did not affect morphological and biochemical endpoints of yellow-legged gull embryos. Our results differ from previous findings showing that experimental manipulation of PFOS concentrations in the yolk of chicken eggs affected embryo hatchability and body growth. However, it is important to emphasize that effective PFOS concentrations used in experiments performed under controlled conditions on developmental and morphological endpoints were considerably higher (μg PFOS g^{-1} egg weight) than those currently found in bird eggs from wild populations and tested in the present study. Thus, based on our results, we conclude that PFOS concentrations that are currently maternally transferred to eggs of the yellow-legged gull do not represent a serious hazard for embryos. However, further investigations on the adverse effects of environmental PFOS concentrations on other endpoints of embryos, as well as on hatchling phenotype, are required to assess the generality of our conclusion. In addition, considering intra- and inter-specific variation of PFOS concentrations in bird eggs, a comparative study of PFOS-induced toxicity in different avian species from different areas and having different diets, implying different exposure levels, would be extremely important to assess the hazards of PFOS to birds under natural conditions. In conclusion, our study demonstrated that the *in ovo* manipulation of contaminant levels in the yolk of yellow-legged gull eggs may represent a useful and promising approach to assess the toxicity of both POPs and emerging pollutants, confirming that the yellow-legged gull is a suitable non-conventional biological model for marine–terrestrial ecotoxicology.

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CHAPTER 14

South American Cowbirds as Avian Models for Environmental Toxicity Testing

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14.1 Introduction

Agriculture and grain production are the economical backbone of many South American countries, with Brazil and Argentina occupying leading positions amongst worldwide producers and exporters. Over the last 20 years, these two countries have widely adopted genetically modified crops and are now front-runners in the production of such cultures, occupying the second and third positions behind the United States in terms of planted superficies.[1] This increased dependence on transgenic crops and the associated no-till culture techniques have resulted in a rapid expansion of pesticide use in the region with Brazil surpassing the United States as the leading world consumer.[2]

Pesticides are toxic chemicals designed to be deliberately released into the environment. Because of the risks associated with the use of pesticides, all countries have established laws and regulations to control the production,

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trade and use of pesticide products. Testing the toxic effects of pesticides on birds is an established part of regulatory assessment in most South American countries. However, even though pesticide products are vastly used in the regional agriculture, no test protocols or guidelines exist for testing effects on native or indigenous bird species. Instead, recommended test species and guidelines for assessing effects are essentially the ones developed for Europe and North America. The lack of testing on native species is clearly a weakness of avian pesticide regulatory assessment in South America. The same could be said of the absence of compulsory testing on passerine species as *Passeriformes* are normally more sensitive than other groups of birds and they represent approximately 60% of all living bird species.

The aim of this chapter is to propose South American cowbirds as candidate passerine avian models for environmental toxicity testing in South American countries. In the first sections, the history and actual situation of avian environmental toxicity testing in South America and the rest of the world are briefly reviewed and compared. The remaining of the text describes the proposed avian model, from its natural life history to its requirements in captivity and during toxicity testing.

14.2 Actual and Historical Use of Birds in Science and Regulatory Toxicology

14.2.1 Birds as Animal Models in Toxicology and Scientific Research

Birds have a long history of serving as animal models in biomedical research. For example, avian embryos are a traditional model in developmental biology and much of what we know regarding vertebrate morphogenesis was first established using avian models.[3] Human cognitive neuroscience and memory research also widely employ avian models, as songbirds provide excellent models for the study of human language learning and the production of speech.[4,5] Useful avian models also exist for a variety of diseases, such as liver diseases, muscular dystrophy, ovarian cancer, and idiopathic pulmonary arterial hypertension.[6–8]

Birds have, in comparison, been considerably less employed in traditional toxicological research. The phylogenetic distance of birds from humans and the fact that classical toxicology historically aimed at predicting effects in humans have resulted in a general preference of mammalian models over avian models.[9] Nevertheless, although differences in drug metabolism exist between birds and mammals, the differences are mostly quantitative rather than qualitative, suggesting that more use could be made of birds in toxicology.[10,11]

Paradoxically, while birds traditionally played a limited role in classic human toxicology, they were at the heart of the emergence and development

of wildlife toxicology and ecotoxicology.[9] Incidents of lead poisoning from ingestion of spent shot by pheasants and waterfowl reported at the turn of the century are amongst the first accounts of wildlife intoxication.[12] Later on, in the 1960s, reports of dramatic declines in wild bird populations and the publication of Rachel Carson's *Silent Spring*[13] brought wide attention to the deleterious effects of toxic contaminants on birds and the environment.[14] These events marked the beginning of governmental activities addressing environmental contamination in a number of different countries and led to the establishment of long-term monitoring programs tracking tissue residues of contaminants in birds and other wildlife.[12,15,16]

14.2.2 Avian Models in Regulatory Environmental Toxicity Testing

The emerging awareness of avian and wildlife toxicology observed in the 1960s also led to the development of laboratory methods for studying chemical toxicity to birds.[17,18] In the 1980s, these efforts resulted in the publication of the first standardized avian protocols by national and international organizations, such as the Environmental Protection Agency of the United States (USEPA), the American Society for Testing and Materials (ASTM), the European Union (EU), and the Organization for Economic Co-operation and Development (OECD).[19–22] These procedures aimed at assessing acute oral, dietary and reproductive effects of chemicals on birds and were the precursors of the tests actually required in most countries for the registration of pesticides and hazardous chemicals.

Nowadays, basic avian testing includes a suite of standardized toxicity tests. In many countries, two acute tests of lethality are required for the registration of pesticide products: a standardized acute oral toxicity test where a single dose is administered orally by either capsule or gavage,[23,24] and a subacute dietary toxicity test where graded concentrations of chemical are presented *ad libitum* in the feed for 5 days.[25] The acute oral test is indicative of the species' sensitivity to the toxic substance, whereas the subacute dietary test provides a measure of the ability to cope with a contaminated diet for a specified duration.[26] In Europe and the United States, a one-generation reproduction test is also mandatory.[21,27] Interestingly, the Avian Acute Oral Toxicity Test guideline 223 released by the OECD in 2010 is an innovative new study design aimed at reducing the number of animals used during testing.[23] The test is based on a staged approach to estimating the LD_{50}, which includes anywhere from one to four dosing stages with varying numbers of test animals depending upon the toxicity of the chemical as calculated at each dosing stage.

In recent years, efforts have focused on the development of an avian two-generation study with endpoints for endocrine disrupter assessment. Birds are particularly vulnerable to endocrine-disrupting chemicals because the biological fitness of a bird can be dramatically affected by very subtle

changes in the normal balance of its endocrine system.[28] A two-generation toxicity test protocol using the Japanese quail (*Coturnix coturnix*) was recently released by the USEPA in 2015.[29] The Japanese quail was selected for the endocrine disruption test because the neuroendocrine regulation of reproduction is known in great detail in this species.[30]

In the case of the other regulatory avian protocols, namely the acute oral test, the subacute dietary test and the one-generation reproduction test, recommended bird species are normally a game bird, such as the northern bobwhite (*Colinus virginianus*) in the United States, or the Japanese quail (*Coturnix coturnix*) in Europe, and a waterfowl, generally the mallard duck (*Anas platyrhynchos*). Since October 2007, the USEPA also requires that avian acute oral toxicity data include a passerine species. The agency, however, states that there is not yet enough information to designate one or more preferred species for passerine acute oral toxicity testing and recommends the use of either the house sparrow (*Passer domesticus*), the zebra finch, (*Taeniopygia guttata*), or the red-wing blackbird (*Agelaius phoeniceus*). The reason for testing passerines comes from the fact that songbirds are often more sensitive to pesticides than the species required by the guidelines, and many of the non-target species exposed to pesticides may be passerine songbirds.[19]

14.2.3 Pesticide Registration and Avian Toxicity Testing in South America

In South America, processes and requirements regulating the use and sale of pesticide products are considerably harmonized amongst countries, with regional groups such as the MERCOSUR (Brazil, Argentina, Uruguay, Paraguay) and the Andean Pact (Bolivia, Colombia, Ecuador, Peru) acting as unifying forces. The equivalence process is prevalent in most countries, which means that registration based on equivalence may be conducted if the pesticide submitted for registration can be shown to be equivalent to a similar pesticide that has already been registered in the country. National and regional regulations are widely aligned with the ''International Code of Conduct on the Distribution and Use of Pesticides'' approved by the Food and Agriculture Organization of the United Nations (FAO).[31] Data requirements suggested in the FAO document originate from those required by advanced regulatory authorities, such as those of Canada, the European Union and the United States of America.

As specifically regards avian toxicity testing, data requirements are similar to those of other regions and both tests of acute lethality are generally required: the standardized acute oral toxicity test and the subacute dietary toxicity test. The one-generation reproduction test is also sometimes necessary.[32,33] Regionally-developed standardized test protocols do not generally exist, so recommended test protocols are the ones developed by countries and organizations from the northern hemisphere.[21,23–25,27] As a

result, recommended test species are normally the ones for which these tests were developed, namely quails and mallard ducks. Compulsory passerine toxicity testing has not yet been implemented in the region.

The acceptability of using non-native species to evaluate risks to the widely diverse Latin American ecosystems was highlighted as a priority question by scientists during the Latin American Global Horizon Scanning and Prioritization Workshop held in Buenos Aires in 2015.[34] The use of native species is normally recommended as it provides greater environmental realism and ensures a more conservative end point for protecting ecosystems.[19,35,36] Ideally, tested species should represent a broad range of sensitivities, habitats, and trophic groups, and be economically, ecologically, and/or recreationally important.[35,37] It is also convenient to select a test species that is abundant and widely distributed as it is more likely to be exposed to pesticides from a variety of use patterns.[19] Other factors to consider in species selection are the amenability to routine maintenance in the laboratory and the extent to which adequate background information is available for these species. Moreover, test species should not be endangered or threatened.[19,37]

14.3 South American Cowbirds' Diversity, Distribution and Life History

Cowbirds are medium-sized New World Passeriformes belonging to the *Icteridae* family. In their natural habitats, cowbirds live in open grasslands where they normally forage amongst grazing animals such as cows, hence the name ''cowbird''. Cowbirds have surged in numbers and range since about the 1900s as a result of deforestation and expansion of agricultural lands, which provided them with new habitats and food sources.[38,39] Cowbirds are omnivorous ground foragers consuming mostly insects, grains and seeds.[40]

Most Icterid species have black as a predominant plumage color, often enlivened by yellow, orange or red. Cowbird species are divided into two different genera according to whether they are brood parasitic or not. Species from the genus *Molothrus*, sometimes referred to as the ''true cowbirds'', are brood parasitic and include the following species: the brown-headed cowbird (*Molothrus ater*), which is the only cowbird species inhabiting North America, the bronzed cowbird (*Molothrus aeneus*), which is found in Central America, the giant cowbird (*Molothrus oryzivorus*) from Central America and the north of South America, the shiny cowbird (*Molothrus bonariensis*), which is found throughout South America and the Caribbean, and finally, the screaming cowbird (M*olothrus rufoaxillaris*) found in north east and central Argentina, south east Bolivia, central Brazil and throughout Paraguay and Uruguay. The baywings are two species of cowbirds that were formerly placed in the genus *Molothrus* but which are now classified in the genus *Agelaioides* because they are non-brood parasitic. They include the pale baywing, which

is endemic of northeast Brazil, and the bay-winged cowbird (also referred to as the grayish baywing) (*Agelaioides badius*), which is found in Argentina, Bolivia, Uruguay, and Paraguay.

Three species of cowbirds are therefore widely distributed in South America and can be considered as potential avian models for environmental toxicity testing: the shiny cowbird, the screaming cowbird and the bay-winged cowbird.

14.3.1 Shiny Cowbird

The shiny cowbird (*Molothrus bonariensis*) is a gregarious passerine bird from the *Icteridae* family. It is a sexually dichromatic species, in which males are all black with an iridescent purple-blue gloss and females are dull grayish brown. Seven subspecies of *M. bonariensis* have been described, which differ markedly in size. The smallest subspecies is *M. bonariensis minimus* (males 39 g; females 32 g) and the largest is *M. bonariensis cabanisii* (males 64 g; females 56 g), with the nominal *M. bonariensis bonariensis* being intermediate (males 56 g; females 45.6 g).[41–43]

Shiny cowbirds are obligate brood parasites (Figure 14.1), which means they lay their eggs in the nests of other species that then raise the cowbird chicks as their own.[44] They are host generalists with more than 200 host species recorded.[45] Female cowbirds reduce the clutch size of the host they parasitize by removing or destroying some of the eggs.[46] Nestling competition between parasite and host chick may be detrimental to the success of the host offspring.[41] Owing to its parasitic lifestyle, *M. bonariensis* represents a threat for bird species already at risk because of habitat loss. Parasitism by shiny cowbirds is believed to have played a role in the decline of endangered yellow-shouldered blackbird and Puerto Rican vireo populations in Puerto Rico.[47,48]

Figure 14.1 Shiny cowbird *Molothrus bonariensis*. Males are all black with an iridescent purple-blue gloss and females are dull grayish brown.

M. bonariensis is common year-round throughout much of South America, except in areas above 2000 m and in extensively forested regions. Frequent in open or semi-open habitats, especially in agricultural areas with patches of trees and shrubs, their range has increased and expanded since about the 1900s as a result of deforestation and expansion of agricultural lands throughout South America and the Caribbean.[38,39] Breeding populations of *M. bonariensis* are now established in South Florida, where they are found year-round, and migrating individuals have been reported in the US south east along the east coast, up to Canada.[49–51] Because of their very large and expanding geographic range, and their very large global population, the IUCN conservation status of the shiny cowbird is rated as Least Concern.[52]

14.3.2 Bay-winged Cowbird

Bay-winged cowbirds (*Agelaioides badius*) are monomorphic and sexes are indistinguishable in the field, although males tend to be slightly heavier (Figure 14.2). With a total length of approximately 18 cm and a mass of 40–50 g, they are dull colored or ashy brownish-gray with rufous or chestnut

Figure 14.2 Bay-winged cowbird *Agelaioides badius*.

wings. The bill, lores and around the eyes are dusky or black, seemingly creating a ''mask''. The tips of the primaries, the inner portions of the secondaries, the tail, legs and claws are black.[53–55]

Bay-winged cowbirds are native to central and southern South America and are found throughout the northern half of Argentina, Bolivia, Uruguay, Paraguay and southern and central Brazil.[56] It is a sedentary species and there does not appear to be any significant, seasonally dependent movement among members of this species; however, local movements result in an increase in flock size in the winter. Banding studies indicate that a member of this species will rarely stray more than 1000 m from its nesting site.[55] They are found in a wide range of semi-open habitats, including scrub and light woodland. As they are generally fairly common, they are considered to be of least concern by IUCN.[52]

An extremely social species, bay-winged cowbirds are commonly seen in small groups. They sing a great deal in all seasons. When flying it frequently utters a peculiar long, loud, and melodious note that may be heard half a mile off on still days. Song is a series of chip notes, trills and whistles.[57] Frequently several males sing together; sometimes females participate.[58] Belonging to the genus *Agelaioides*, this species is not a brood parasite. The breeding season generally lasts from November through March. Owing to their semi-colonial nesting habits, mated pairs of bay-winged cowbirds usually receive assistance in provisioning and nest defense from other adults.[59,60]

14.3.3 Screaming Cowbird

Screaming cowbirds (*Molothrus rufoaxillaris*) are sexually monomorphic (Figure 14.3). They are shiny black with a purple sheen all over and have rufous axillars that are almost impossible to discriminate in the field. About 19 cm long and with a weight of 50–60 g, the bill is short compared to other cowbirds, as implied by their vernacular Spanish name: tordo de pico corto.[40] The screaming cowbird is a specialist brood parasite, with only three host species known so far. The primary host is the bay-winged cowbird, but the screaming cowbird also parasitizes the nests of the chopi blackbird (*Gnorimopsar chopi*)[61,62] and the brown and yellow marsh bird (*Pseudoleistes virescens*).[63]

The distribution of the screaming cowbird closely overlaps that of its principal host the bay-winged cowbird in central and southern South America, from Bolivia and southern Brazil to central Argentina. Within their range, screaming cowbirds are common in numerous habitats, including open woodlands, grasslands, modified agricultural, pastoral and suburban landscapes.[40] They are quite sedentary and are found within the same area throughout the year. Minimal home ranges for screaming cowbirds are between 15 and 25 ha.[61] As they are generally fairly common, their IUCN conservation status is rated as Least Concern.[52]

Figure 14.3 Screaming cowbird *Molothrus rufoaxillaris*.

14.4 Cowbirds as an Avian Model for Environmental Toxicity Testing

The decision to select one or another of the three cowbird species that are potential candidates for use as avian models, namely the shiny, screaming and bay-winged cowbirds, will depend on different factors, such as the relative local abundance of the three species and their resulting ease of capture; or on the specific objectives of the study and whether or not it is necessary to sexually differentiate tested animals. For example, shiny cowbirds present the advantage of sometimes occurring in large flocks and that sexes can be differentiated, but the existence of seven subspecies may complicate their use in locations where subspecies coexist.

Although experts generally recommend the use of captivity-reared birds owing to animal welfare considerations and disease control,[23] the use of wild-caught species is judged acceptable and tolerated in the case of passerine species, as cultured supplies of such species are not widely available and little information is available regarding the establishment and performance of breeding stocks.[24,64,65] The situation is similar in the case of

South American cowbirds and the use of wild-caught animals will have to be tolerated until breeding protocols and stocks can be established. Antecedents of captive breeding with the brown-headed cowbird are encouraging in this sense.[66]

Cowbirds are amenable to captivity as they quickly become tame and show all of the displays of free ranging birds, with displays in captivity and in nature being done in similar contexts.[67] A great number of studies have used the North American brown-headed cowbird to study behavioral or reproductive aspects, such as dominance, pairing, courtship, and fecundity.[68–72] Brown-headed cowbirds have furthermore been used in various environmental toxicology studies.[73–76] While the number of scientific studies to have used South American cowbirds is fairly restricted, there is no reason why these species should respond differently to captivity, and the few behavioral and toxicological studies to have used them successfully support this view.[77–79]

14.5 Methods for Maintaining and Using Cowbirds in the Laboratory for Environmental Toxicity Testing

This last section describes the methods we currently use for capturing, acclimating, maintaining and testing cowbirds. We have successfully used the following set of procedures to obtain, and maintain bay-winged cowbirds for use in acute and subacute exposures.[79,80]

14.5.1 Capture and Transport

Cowbirds are captured using walk-in funnel traps. Funnel traps consist of a funnel leading into a trap. Birds walk through the funnel into the trap, lured by bait, and they are most often unable to exit. The traps we use are circular with a diameter of 1 m and a height of 15 cm (Figure 14.4). They are built of metal wire mesh fixed over a metal frame. The diameter of the mesh is 2 cm. Four funnel entrances are located on opposite sides of the trap. The exterior entrance of the funnel has a diameter of 15 cm and its interior exit has a diameter of 10 cm. The most effective bait is bread as cowbirds detect it much more rapidly than seeds. A few pieces of bread are offered in the funnels, while a generous amount of bread is located in the center of the trap. The efficiency of the trap is reduced if too much bait is available on the outside, as cowbirds will not enter. Baiting the location a few days before placing the trap is recommended to ensure a larger number of birds visiting.

The health and well-being of the birds should be a primary concern during all phases of capture. To prevent injuries caused by stress and because cowbirds can sometimes escape from the trap, birds should remain in the traps for as little time as possible. For this reason, it is recommended that

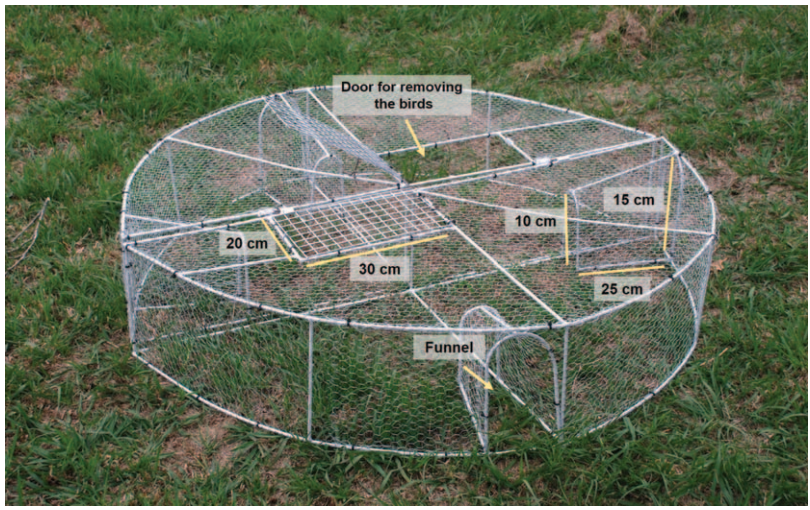

Figure 14.4 Diagram of the walk-in funnel trap used to capture cowbirds. The trap has a diameter of 1 m and a height of 15 cm.

operative traps be controlled every 15–30 minutes. It is useful to first block every funnel with cloth or other similar materials when approaching the trap to recover a captive bird as this prevents the animal from escaping. Captured birds should be transported to their final housing facilities as soon as possible. To reduce stress, it is recommended to cover transport cages with a blanket, so birds are in the dark. The recommended density for transport is five birds per m^2. Care must be taken when handling birds as they may carry diseases that can affect humans and other animals. Routinely wash hands after handling all birds; the use of a face mask is recommended. Wild bird capture is an activity that is strictly controlled in most countries. Make sure to obtain all the required local, state, provincial and federal permits. To avoid interfering with reproduction, cowbirds should only be captured outside of the breeding season, which generally ranges from September to March in South America. This means that captures should be concentrated between the months of May and August.

14.5.2 Housing, Acclimation and Feeding

Whenever experimental animals are used, minimizing pain and distress should be as important an objective as achieving the experimental results. Adequate environmental and housing conditions are essential for bird welfare, so care should be used in maximizing and closely controlling the different housing variables. In our laboratory, cowbirds are housed in a climate-controlled room at 23 ± 2 °C and 10 : 14 hours light:dark photoperiod. Dimension of the cages are $60 \times 40 \times 40$ cm. As cowbirds are social, it is usually best to locate at least two individuals per cage. In our experience,

Figure 14.5 Bay-winged cowbird *Agelaioides badius* in captivity.

cowbirds should be offered no less than 800 cm^2 of floor area per bird, which corresponds, in our cages, to a maximum of three individuals per cage (Figure 14.5). It is essential for the cage to contain plenty of perches as cowbirds like to fly from one to another. Individual cages and the room in general must be cleaned daily.

It is essential to closely monitor new arrivals in order to detect abnormal anti-social behaviors. One common such behavior is when a dominant bird injures other birds in the pen by repeatedly pecking them, sometimes causing their death. In these cases, it is best to separate involved birds and locate them in a cage where density is lower. This normally solves the problem. In a few rare cases, the trouble bird has to be kept on its own as its anti-social behavior never stops. A clear sign that birds have acclimated is that they normally start singing 3 to 4 days after arrival. Bay-winged cowbirds acclimated to their housing conditions sing frequently throughout daylight hours. A much appreciated cage enrichment consists of the addition of a water bath at the bottom of the cage. Cowbirds also like to pick on small sticks or pieces of paper if available.

Cowbirds are fed a commercial seed mixture for canaries, which is composed principally of millet and canary seeds, and is enriched with vitamins. Every other day, this seed meal is supplemented by covering it with a thin layer of crushed commercial food for insectivorous bird. Food is offered *ad libitum* but it is important to replace the seed mixture daily as cowbirds peel the millet seeds and leave the shells in the container, making it look full. Water is offered continuously through a water dispenser. The water in the dispenser is renewed every day.

14.5.3 Acute Oral Toxicity Testing

For oral gavage, the use of a nasogastric tube with 2.0 mm diameter embedded in petrolatum is recommended. The volume administered should

be no more than 500 μL to avoid regurgitation of the test solution. Almost no regurgitation was observed using such a volume, but the regurgitation rate augmented if greater volumes were administered. To control for regurgitation, a red food coloring was added to test solutions so any regurgitate would be conspicuous on the white paper placed at the base of the cage. Birds were euthanized in a carbon dioxide gas chamber. The chamber must be clean and without any CO_2 when incorporating the animal. The flux of CO_2 should be 10% of the chamber volume per minute. Cleaning the chamber after every use to eliminate odors reduces stress to the birds.

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CHAPTER 15

Epilogue and Final Remarks

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Nowadays, the growing impact of anthropogenic activities generates huge quantities of toxic residues that can have direct or indirect detrimental effects upon the quality of our environment. These increased jeopardizing effects can exert short-, medium- and long-term consequences that affect human and environmental health in general, but are also capable of reducing the biodiversity of native flora and fauna, which will, in turn, encourage the resistance and emergence of new pests and diseases.

This book, *Ecotoxicology and Genotoxicology: Non-traditional Terrestrial Models*, intends to provide an overview and relevant examples to stimulate practical discussions on the use of non-conventional biotic matrices within the scientific challenges faced by the ecotoxicology and genotoxicology academic world. Furthermore, the book endows relevant tools that may be of use in the implementation of decisions leading to actions that will hopefully reduce the environmental health risk against environmental factors that may adversely impact human health or ecological balances.

We aimed to compile information from a diversity of sources into a single volume. The rationale is to give some real-life examples in order to widen the

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concept that the use of a non-conventional animal models, far from being a scientific conundrum, may instead provide real answers to some of the actual problems the whole planet is dealing with. Concomitantly, these real examples extend concepts of hazardous factors to living species that may stimulate new research ideas and trends in the relevant fields.

In *Terrestrial Invertebrates as Experimental Models*, readers will find five chapters (Chapters 1–5) with background information about the nature of some environmental pollutants and some of the most versatile and validated methods of analysis. These depict different organisms as well as specific examples of the use of soil microfauna (less than 0.1 mm), mesofauna (0.1 to 2 mm), macrofauna (2 to 20 mm) and megafauna (larger than 20 mm). The majority of soil invertebrates can be found in the first three groups while megafauna is only represented by some larger sized invertebrates, including Chelicerata, Mollusca and Annelida. The first chapter provides an overview of general considerations for soil invertebrates, such as their representativeness within the Animal Kingdom, and their classification into groups, as well as some morpho/physiological characteristics and habitat requirements. The importance of the role of soil fauna in terrestrial ecosystems is reported, highlighting the use of invertebrates in soil ecotoxicological assays and thus in terrestrial environment studies. The chapter lists species of soil invertebrates currently used in standard laboratorial ecotoxicological assays and proposes new standard and alternative test species of interest in soil ecotoxicology testing. The advantages and disadvantages of their use are also mentioned. The uses of some taxonomic groups like Annelids, *e.g.*, earthworms and potworms, Arthropods, including Crustaceans, *e.g.*, Isopods, Hexapods, *e.g.*, Collembolans, and a few others, with recognized potential for ecotoxicological studies are quoted as examples of this approach. Chapter 2 provides a review highlighting that safe and efficient crop production depends on good ecological status, which includes a typical biodiversity of communities of soil organisms. It is also suggested that pesticide registration should involve an environmental risk assessment to assure that the general goal of protection of biodiversity is achieved. Specific risks to non-target soil communities require advanced methods in tiered ''multispecies higher-tier'' approach test systems on a semi-field level. A well-described and -understood test system could serve as a surrogate reference tier for the calibration of the risk assessment of pesticides. Chapter 3 documents the use of a non-conventional annelid species to evaluate the genotoxicity potential of real-world environmental contexts accurately and in a high-throughput, low-cost fashion. *Aporrectodea longa*, an abundant species of earthworm that lives in close contact with soil systems, could be adapted as a sentinel organism to investigate spatial and temporal exposure effects. For genotoxicity evaluation, authors have adapted the single cell gel electrophoresis (Comet assay) employing coelomocytes from *A. longa* specimens exposed to different xenobiotics. Amendment of soil samples with a ^{14}C-labelled compound allowed organism uptake to be quantified. This showed that *A. longa* either ingests or dermally absorbs environmental

contaminants in soil. The chapter pinpoints that *A. longa* can be employed as a sensitive indicator of environmental contaminants and the non-conventional model has great potential for identifying environmental contamination, especially for use in monitoring of land remediation. Chapter 4 presents field and laboratory methods for using the land snail *Helix aspersa* to characterize genotoxic effects of soil pollutants. The suitability of *H. aspersa* among terrestrial invertebrates is highlighted, since it can accumulate different classes of chemicals and fulfills the criteria of relevant species for monitoring contaminated soils. Chapter 5 reviews the use of spiders as important regulators of insect population sizes in meadow, forest and agricultural ecosystems owing to their polyphagy, low nutritional selectivity and high hunting activity. In anthropogenically changed environments, the occurrence and hunting efficiency of these obligatory predators depend on their ability to tolerate excess amounts of xenobiotics introduced to organisms *via* ingestion or contact. It is also well documented that spider sensitivity to environmental xenobiotics is both species- and sex-specific. Analysis of changes in selected cellular parameters characteristic of the response to stressing factors may allow the identification of defence strategies triggered by spiders as induced by different environmental stressors, and the prediction of their ability to survive in polluted sites as well as recolonise anthropogenically changed areas.

In the second section of this book, *Terrestrial Vertebrates as Experimental Models*, readers will find nine chapters (Chapters 6–14) also dealing with background information about the nature of some environmental pollutants, some new strategies and the most useful and validated worldwide methods of analysis. The use of different organisms as well as specific examples of several taxa, including amphibians, reptiles and birds is taken into consideration. Chapter 6 describes the usefulness of the analysis and evaluation of Neotropical anurans' internal pigmented cells, such as melanomacrophages and melanocytes and systemic responses to environmental contaminants. Data clearly reveal that the amount of melanin among these pigmented cells varies upon both type of contaminants and group of species analyzed. It is clearly indicated that environmental contaminants alter the internal melanin content in anurans. Furthermore, it also seems evident that internal melanin is an important pigment capable of protecting exposed organisms against possible damage caused by environmental contaminants. Chapter 7 presents an overview of the use of European terrestrial life-stages of amphibian species as model organisms. Most species occurring in the cultivated landscape have a high risk of coming into contact with pesticides. This is achieved by exposure to residues on food (oral uptake) or by dermal uptake (owing to direct over-spraying or contact with contaminated plant material or soil). Although species-specific differences in sensitivity are well known in amphibian toxicology, only 8% of European amphibian species have ever been used as test organisms in toxicological studies and urodele species are especially underrepresented. Furthermore, birds and mammals, which are currently serving as surrogate species for pesticide toxicity to

terrestrial life-stages of amphibians, dramatically differ in their biology and ecology. In particular, the fast dermal uptake of xenobiotics by amphibians questions the unique use of data obtained by oral administration of pesticides to surrogate species in pesticide approval use by the authorities. Authors accurately highlight that indirect effects, mostly owing to a strong reduction of food resources due to pesticide applications, as well as the presence of adjuvants in pesticide formulations with known or suspected jeopardizing effects, are not always adequately considered in current risk studies. Continuing with the problems previously posed, Chapter 8 offers a review of the global biodiversity crisis of amphibian populations as the most threatened and rapidly declining vertebrate group. Modern agricultural expansion and intensification is increasingly involved in the amphibian decline owing to pesticide contamination coupled with habitat loss and fragmentation. In Argentina, the Pampas consist of a vast grassy plain rich in rivers and ponds where agriculture and animal husbandry dominate. Over the last 40 years, the region has experienced a great expansion of the cultivated surface as well as an intensification of production through the use of fertilizers and pesticides. The gradual transformation of the landscape is likely to impact upon the regional herpetofauna. As amphibians are key elements of food chains, whole ecosystems may eventually be altered by amphibian declines. Five amphibian species as potential models are suggested and illustrated. They include *Leptodactylus latinasus*, *L. latrans*, *Hypsiboas pulchellus*, and *Rhinella fernandezae* in conjunction with *R. dorbigny*, and *R. arenarum*. *R. fernandezae* and *R. dorbigny* are also considered as alternative species. The characteristics and life history of these species are described, as well as current antecedents surrounding their use as bioindicators and biomonitors; a very useful and interesting model indeed, as not many species can serve this dual purpose. Continuing with practical examples of new non-conventional species, Chapter 9 presents *Odontophrynus cordobae* as a valid anuran model for laboratory and environmental monitoring studies. This is an endemic species to central and northern Argentina. It combines proper biological and ecological features for laboratory and environmental monitoring studies and handling, as the species is abundant and easy to collect, simple to manipulate under laboratory conditions and presents few difficulties with blood extraction using minimally invasive techniques. According to the literature, *O. cordobae* has a greater sensibility to genotoxic agents when compared to other amphibian species. Genotoxicity tests in their peripheral blood cells provide scientific support for *in situ* studies of the potential risks produced by environmental exposure to genotoxic agents. Finalizing the chapters committed to the use of amphibians, Chapter 10 presents the use of the direct-developing frog *Eleutherodactylus johnstonei* as an innovative and promising model organism in terrestrial ecotoxicology. Standard guidelines for collecting, housing and rearing this frog under laboratory conditions are given. The chapter also provides an overview of candidate end-points and ecotoxicological tools for the future use of this species in ecotoxicological

studies. Further ecotoxicological research focused on *E. johnstonei* as a model organism would be a welcome addition to our understanding of agrochemical exposure in terrestrial amphibians. This is followed by two chapters employing other Chordata such as reptiles as non-conventional models. Chapter 11 emphasizes the use of an endemic species to South America, the lizard *Salvator merianae*, the black tegu. This Squamata lives in a variety of environments, including open spaces of primary and secondary forest, savannah with thorny bushes, tropical rain forests, riverbanks and sandy coastal areas. It is also found in disturbed habitats, including roadsides and agricultural areas. Results of genotoxicity studies performed in neonates and adults of the species when exposed *in vivo* or *in ovo* to commonly used agrochemicals suggest that *S. merianae* can be considered as a good indicator for genotoxicity assessment of contaminants, at least when Comet assay and micronucleus tests are employed as biomarkers. Chapter 12 introduces another Squamata species, *Podarcis sicula*, the Italian wall lizard, ruin lizard, or Istanbul lizard, as an experimental model for emerging contaminants. An advantage of using *P. sicula* as a bioindicator of oestrogen pollution is that its full reproductive cycle is well known. Males, in particular, are excellent sentinels since they are able to synthesize vitellogenin when exposed to an oestrogenic environment; hence, the precursor protein of egg yolk is commonly used as a biomarker of pollution from oestrogenic compounds. Data collected using *P. sicula* for monitoring soil health status in both intensive and organic farming, as well as for the study of tissular and cellular damage following environmental contamination by oestrogenic compounds, is presented and discussed. The following two chapters describe the validity of using four species of birds as non-conventional models. Chapter 13 reviews the applicability of eggs from a non-conventional model organism for marine–terrestrial ecotoxicology studies to investigate the toxicity of emerging environmental pollutants. The chapter summarizes the effects of environmental concentrations of perfluorooctane sulfonate on embryos of a Charadriiform, the yellow-legged gull (*Larus michahellis*), on diverse phenotypic traits, including morphometric and biochemical endpoints by an *in ovo* manipulation approach. The authors clearly show that the yellow-legged gull is a reliable model organism for ecotoxicology and its eggs are a useful tool to monitor the levels and the toxicity of emerging environmental pollutants. Last but not least, Chapter 14 reviews the fact that although pesticide products are vastly used in South American agriculture, no test protocols or guidelines exist for testing effects on native or indigenous bird species. The recommended test species and guidelines essentially mirror those developed for Europe and North America. Avian pesticide regulatory assessment in South America does not require testing on passerine species although *Passeriformes* are normally more sensitive than other groups of birds and represent approximately 60% of all living avian species. The chapter proposes South American cowbirds as candidate passerine avian models for environmental toxicity testing in South American countries. Three species of cowbirds are widely distributed in the region and

can be considered as potential avian models, namely the shiny cowbird (*Molothrus bonariensis*), the screaming cowbird (*Molothrus rufoaxillaris*) and the bay-winged cowbird (*Agelaioides badius*). This chapter briefly reviews and compares avian environmental toxicity testing in South America and the rest of the world, also describing requirements for maintaining cowbirds in captivity and using them in toxicity testing.

As well as the purely terrestrial and the purely aquatic animals, there are many borderline species. There are no universally accepted criteria for deciding how to label these species, thus some assignments are disputed. For some taxa, the classification of an animal species as ''terrestrial'' or ''aquatic'' is often an obscure process and becomes a matter of judgment.

Among the examples presented in this book, this is the case for the Amphibia anuran species. Amphibians are intermediate in some ways between the fully aquatic fishes and the terrestrial amniotes. From an evolutionary point of view, amphibians have undergone a remarkable adaptive radiation in their attainment of independence from water and colonization of land. This living group exhibits one of the greatest diversities of modes of life history than any other group of vertebrates. The life cycle of most amphibians includes laying anamniotic eggs in water, followed by an aquatic larval stage, a period of metamorphosis from larva to juvenile, and an adult stage that can occur in both aquatic and terrestrial habitats. In other words, they are somewhat dependent on water for reproduction because the egg is never protected by a hard shell. According to this concept, anuran species quoted in this publication are employed as an experimental model involving the use of the terrestrial stages of the species. However, it should be mentioned that two chapters have included both aquatic and terrestrial stages of development. The reason for this line of thought relies on the fact that most of the conclusions have been reached when adults of the species have been employed for analysis.

In spite of dealing with many diverse topics, we have tried to compile this ''wealth of information'' into two major parts for the sake of clarity and order. Firstly *Terrestrial Invertebrates as Experimental Models* and secondly *Terrestrial Vertebrates as Experimental Models* take into consideration whether invertebrate or vertebrate taxa of one or more populations of a living organism or organisms have been selected as experimental matrices, respectively.

Without running the risk of being repetitive, we would like to recap on some important concepts as previously mentioned in *Non-Conventional Animal Models in Ecotoxicological and Genotoxicological Studies: Aquatic Models*, by the same editor. We strongly recommend the perusal of both volumes, which do not cover overlapping subjects, in order to gain the full benefit of this series and have a holistic and stimulating approach to the matter.

Many scientists feel that the time has come for a shift in emphasis away from Basic research towards Applied science. While Basic research is needed in order to shed more light on the fundamentals, Applied research is

required in order to find solutions to the many problems the world faces, *e.g.*, overpopulation, excessive use of the Earth's natural resources and pollution.

New emerging studies in several reputable publications [*e.g.*, Worldwide Trends 1975–2015, *The Lancet*, 2016, **384**(10064), 37–55] of common worldwide human diseases such as arterial hypertension, among others, seem to suggest that not only classical and lifestyle factors should be addressed in the search for adequate treatment. From a medical perspective, it is not only a disease of affluence. One in eight deaths worldwide is owing to this condition and its corresponding factors (heart and kidney disease, and stroke). These studies seem to suggest that a closer look at other, until-now unrelated, factors is required. Early-life nutrition and exposure to air pollution, heavy metals and even noise are implicated factors that may push blood pressure up later in life. Thinking out of silos and cross-linking medical with applied science will lead to a faster pace of understanding of all the factors related to the emergence of a disease and thus finding a cure.

The chapters included in this book are a mere enumeration of some practical examples. There are many more species that can be used as experimental models and the list should be expanded by different research groups and academics all over the world. We hope that many more scientists realise that it is more important to tackle subjects related to the status of the environment using autochthonous, non-target species that are truly exposed to locally used xenobiotic agents. On the other hand, this research plan would also increase the chances of independent scientists and research institutions getting access to grants and attracting the attention of local authorities, who would be more interested in financing projects that really are within their sphere of political interest and pride.

Many researchers have contributed to the publication of this book. We hope that it serves as a herald in order to bolster enthusiasm for the use of native, easily available local species in order to widen our knowledge on the subject. Last but not least, we would like to especially thank the authors for their positive response, their time, contributions and feedback, making possible the compilation of this book.

Subject Index