Work package 3.3 description:

Toxicokinetic modelling (first 18 month plan)

Work package number	WP 3.3	**Start date or starting event:**		Month 7			
Participant id	VU	NERC	DSTA	UJAG	UA	WRcNSF	LMC
Person-months per participant	12	6	3	9	3	5	12
Participant id	UFZ						
Person-months per participant	12						

Objectives

The objective of WP 3.3 is to define the role of toxicokinetics in governing the responses of diverse taxa with different physiologies to exposure to single compounds, mixtures and other environmental stressors. To realise this objective, the following aims have been defined for the first phase of the work package:

1.Further refinement of analytical methods for the determination of selected chemicals in the test organisms used to determine uptake and elimination kinetics.

2. Determining uptake and elimination kinetics of selected chemicals in aquatic and soil organisms upon exposure to single chemicals and mixtures.

3.Determining the impact of chemicals in a mixture on each others uptake and elimination kinetics in aquatic and terrestrial organisms.

Description of work

During the first 18 months, the work will focus on the determination of uptake and elimination kinetics of selected single chemicals and mixtures in a series of aquatic and soil organisms, representing different taxonomic groups and different physiologies.

The decision of which single chemical, chemical mixture and multiple stressor scenarios will be tested will be taken on the basis of the output of WP 1.2 and through detailed assessment of the results of the work planned in WP 3.1. In the initial phase, this WP will mainly focus on establishing the methods of determining the kinetics in the selected test organisms, by focusing on a few selected chemicals and their mixture(s). At a later stage, emphasis will be placed on mixtures of chemicals or of chemical and non-chemical stressors that showed typical interactions in toxicity experiments performed within WP 3.1 and WP 3.2.

Test organisms used in this WP will include Oligochaetes (NERC), Collembola (VU), Carabidae (UJAG), daphnids (UA) and molluscs (DSTA). The first three test organisms will be exposed in soil, while the latter two will be exposed in water. The experiments will include an uptake phase, during which the organisms are exposed to non-toxic concentrations of single chemicals or mixtures. During this phase, at regular time intervals test animals will be sampled and analysed for accumulated chemical concentrations. After a certain uptake period, typically 2-4 weeks depending on the organism and test substrate, animals will be transferred to non-treated test substrates and elimination will be determined by analysing test animals at regular time intervals. Colleagues working in WP 4.1 will provide methods for the modelling of uptake and elimination kinetics. These models will be applied to the single chemical exposures, and to determine modification of

uptake and/or elimination kinetics by exposure to mixtures of chemicals

Colleagues involved in WP 2.2 will provide methods for the analysis of test chemicals in the test substrates and in the test organisms. In collaboration with the other partners involved in this WP, WRcNSF will take the lead in further refining these test methods to make them applicable for measuring chemical concentrations in the test organisms. The resulting Standard Operating Procedures provided by WP 2.2 or generated in this WP, will be used by all partners to perform chemical analysis for their experiments. To ensure proper implementation of the methods of chemical analysis, training of partners involved in this WP will take place in the laboratories of colleagues of WP 2.2 and WRcNSF will provide advice and technical supervision. Where possible, partners involved in this WP will co-operate in the chemical analysis, with partners most experienced in the analysis of a certain type of chemical taking care of the analysis of those chemicals also in samples of the other partners. A plan for the mutual exchange of protocols, reference materials and samples will be prepared to ensure optimal use of available expertise and facilities.

Uptake and elimination will not only be related to total (measured) chemical concentrations in the test substrates, but also to (bio)available concentrations. Available concentrations will be measured in close co-operation with and using methods provided by colleagues working in WP 2.2.
Data of this WP will be fed into the NOMIRACLE database and delivered to WPs 4.1 and 4.2 to form a basis for risk assessment analysis and the development of QSARs for mixture interactions at the organism and molecular levels under complex exposure conditions (in mixtures and upon exposure to non-chemical environmental stressors). From this basis we will then be able to move on to later work in NOMIRACLE. This will include collecting data on the uptake and elimination kinetics of a series of selected chemicals in the selected test organisms, upon single and mixture exposure and under the influence of other environmental stressors (to be defined by RP 1 and in WP 3.2). Additionally we will assess the degree of modification of chemical uptake and elimination kinetics in mixtures and by non-chemical environmental stressors. To develop predictive methods for the hazardous effects of indoor exposure on human health, targeted QSAR investigations will be performed to derive a mapping between VOC compound effect profiles on biotest systems and epidemiological findings about the exposure-related human health status.

Deliverables

D.3.3.1 Standard Operating Procedures for the analysis of concentrations of selected chemicals in the different test organisms (15 months).
D.3.3.2 Preliminary report on the uptake and elimination kinetics of two selected chemicals and their mixtures in different test organisms, upon exposure in soil or water (18 months).

D.3.3.3 Literature report on the toxicokinetics of chemicals in mixtures as a first step towards the development of a QSAR for mixture interactions (18 months).

Milestones[15] and expected result for first 18 months

M.3.3.1 Agreement on the first set of chemicals for toxicokinetic analysis (9 months).
M.3.3.2 Methods for the analysis of selected chemicals in biological tissues, to be described in the form of Standard Operating Procedures that can be used by all partners in this WP (15 months).
M.3.3.3 Identification of the suitable compartment models for each of the species in collaboration with partners in WP 4.1 (12 months).
M.3.3.4 First data on the uptake and elimination kinetics of two selected chemicals in the selected test organisms, upon single and mixture exposure (18 months).
M.3.3.5 Review of literature data on mixture interactions affecting the uptake and elimination kinetics of chemicals in aquatic and terrestrial organisms (18 months).

[15] Milestones are control points at which decisions are needed; for example concerning which of several technologies will be adopted as the basis for the next phase of the project.

Work package 3.4 description:

Molecular mechanisms of mixture toxicity (18 month plan)

Work package number	WP 3.4	**Start date or starting event:**		Month 7			
Participants id	DSTA	NERC	UFZ	WU	UWC	UCAM	EKUT
Person-months per participant	8	8	7	6	9	9	6
Participants id	UA						
Person-months per participant	9						

Objectives

Because of the potential importance of mode of action for understanding combined stressors effects this work package will elucidate the conserved and distinct molecular changes that underpin the systemic response of species to chemical mixtures (WP 3.1) and multiple stressors (WP 3.2). Ultimately we will derive biomarkers of combined exposure and effect that will be validated in field studies in the later stages of WP 3.2. Specific objectives for the first 18 months of the work package are set out below.

1. Develop a co-ordinated approach that exploits methods for measuring gene and protein expression and function for mode of action assessment. We will start by optimising protocols and conducting exchange/training visits to transfer methods between human model systems and environmentally relevant species and if applicable vice versa. Standard operating procedures will be made available to the Consortium and ultimately the wider community as a handbook. Baseline measurement will then be made in each species-system under optimal conditions.

2. Establish mode of action for prioritised single compounds from literature and past work. If no relevant information is available, mechanisms will be determined experimentally. Initially, comparative analysis of molecular response patterns will be undertaken to elucidate the conservation of stress responses for compounds with well known modes of action. On this basis we will define an optimum strategy for the use of transcriptomics, proteomics, metabolomics, targeted biochemical and pattern recognition methods for mode of action assessment.

3. Use biochemical/molecular tools to assess (confirm) that chemicals with similar modes of action whose toxicity can be described by concentration addition act through a single response cascade.

Description of the work

To optimize the use of resources within NOMIRACLE, information on modes of action will first be collected from literature. If there is no information available relevant to the biological system in question, public toxicogenomics and metabolic pathways resources (e.g. KEGG metabolism, and *pathDB* metabolic pathway databases) will be screened and any available data used to elucidate mechanism(s). If this screening yields no data, mode of action will be investigated experimentally in the most relevant biological system. These include human cell lines (UFZ), rodent (NERC, UCAM), fish (EKUT, UFZ), annelid worm (NERC, UWC), mollusk (DSTA), nematode (UWC, WU), *Daphnia* (UA) and a single cell organism (DSTA). The work concentrates on animals in order to increase the relevance to human metabolic systems and ultimately human health.

The biochemical/molecular toolkit for mode of action assessment

The experimental approach used to establish mode of action (and ultimately interaction) comprise

both comprehensive and targeted methods. DNA microarrays for zebra fish (UFZ), nematode (WU, UWC), earthworm (UWC), *Daphnia magna* (UA), mussel (DSTA) and *Dictyostelium* (DSTA) will be used to identify changes in gene expression. Other gene discovery techniques will also be employed (UWC, UA). Proteomics will use electrophoresis and chromatography separation with mass spectroscopy (DSTA, UA, UCAM, NERC). For metabolomics, ^{1}H NMR spectroscopy, GC-MS and LC-MS based approaches (UCAM, NERC) will be used. To supplement screening techniques, detailed histopathological, biochemical, physiological, and molecular genetic analyses will be made (see Section B4 for full details) (DSTA, NERC, EKUT, UA). After data acquisition, pattern recognition will be used to interrogate the datasets, allowing us to identify metabolic and gene responses characteristic of specific exposures. Comparative analysis (co-ordinated by DSTA) will establish whether mechanisms are unique to species or common between taxa.

Developing a co-ordinated approach for mode of action and interaction assessment
Prior to the experimental phase, a comprehensive harmonisation process, including a program of exchange training visits, will be undertaken to optimise procedures for the measurement of stress responses. These adapted protocols will be compiled on the NOMIRACLE web site.

Establishing compound mode of action
In the initial phase of WP 3.4, the biochemical/molecular toolkit will be used to confirm mechanisms for compounds with a well known mode of action. These could include for example, cholinesterase inhibiting pesticides, respiratory inhibitors, and blockers of metabolic biosynthesis. Completion of this work will establish the ability to identify mode of action and will suggest refinements for optimal use of the toolkit for chemicals where mode of action or mixture interaction is not known or not relevant to a particular species.

Mode of interaction assessment for similarly acting compounds
After testing for single compounds, the biochemical toolkit will next be used to identify modes of interaction for chemical mixtures and later multiple stressors. In the first 18 months these investigations will seek to confirm that chemicals with a known similar mode of action whose toxicity can be describe using the addition model act through a similar response pathway. Completion of this study with similarly acting chemicals will refine our approach for later analyses of mixtures that i) have dissimilar modes of action (including combinations of chemical and non-chemical stressors), ii) show consistent deviations from the addition and independent action models.

Deliverables

D.3.4.1 Repository of protocols for specific and global profiling of stress response pathways in human cells, mammalian models and environmentally relevant species (12 months).

D.3.4.2 Completion of baseline analysis of response profiles for single stress response systems and global profiling responses in the selected experimental systems under ideal conditions (18 months).

D.3.4.3 Datasets of analyses on the effects of two compounds with a known specific mode of action and one additive mixture on global and specific biochemical responses (18 months).

Milestones[16] and expected result for first 18 months

M.3.4.1 Agree on format for collation of technical guidance and standard operating procedures onto NOMIRACLE web site (9 months).
M.3.4.2 Decide initial series of research training visits (12 months).
M.3.4.3 Complete cataloguing of available information on mode of toxic action for prioritised chemicals in all species (18 months).

[16] Milestones are control points at which decisions are needed; for example concerning which of several technologies will be adopted as the basis for the next phase of the project.

M.3.4.4 Identify two compounds with known specific mode of action to be used to confirm the intra-species comparability of biochemical assays (12 months).
M.3.4.5 Harmonise testing procedure and agree protocols for exposure and sample collection and handling with partners involved mixed stressor studies in WP 3.1 and 3.2. (12 months).
M.3.4.6 Complete assessment for two compounds with a single specific mode of action (all partners) (18 months).
M.3.4.7 Review and ultimately refine the biochemical toolkit for mode of action and interaction evaluation (18 Months).

Work package 4.1 description:

New concepts and techniques for probabilistic risk assessment (18 month plan)

Work package number	WP 4.1	**Start date or starting event:**	Month 0	
Participant id	VU	DESUN	USOUTH	EPFL
Person-months per participant	12	13	18	18

Objectives

WP4.1 aims to develop new concepts and techniques for probabilistic risk assessment that are scientifically sound and practicable for management purposes. The specific objectives during the first eighteen months of the NOMIRACLE project are:

- Separation of true uncertainty and interindividual variability in risk predictions of an integrated human exposure model.
- Extension of the DEB theory for single compounds in order to derive a probabilistic NEC for a mixture of two known compounds.
- Harmonisation of the analytical frameworks for meta-analyses of human and ecological toxicity data.
- Derivation of probabilistic uncertainty factors (UFs) for inter-individual and interspecies differences based on pharmacokinetic and pharmacodynamic data for exposure to single compounds and chemical mixtures that are handled by major phase I and phase II metabolic pathways.
- Development of new methods for comparative risk assessment by integration of mixture toxicity and multiple stressors (i.e., comparison of toxic stress, eutrophication and acidification).

Description of work

Separation of uncertainty and variability (DESUN)

The integrated human exposure model NORMTOX (Ragas & Huijbregts 1999) predicts the risk of human exposure to chemical contaminants from all relevant environmental exposure routes (air, drinking water, surface water, food, soil and dust). During the first stage of WP4.1 (starting after month 6), the influence of true uncertainty in the risk predictions of NORMTOX will be separated from the influence of interindividual variability by means of nested Monte Carlo simulation. To perform these calculations, detailed data will be gathered on contaminant concentrations in relevant environmental media and on human consumption and activity patterns. These data will be analysed to derive the relevant statistics of the input parameters for the nested Monte Carlo simulation. At first, detailed risk predictions will be produced for exposure of the Dutch population to a selected set of pesticides. If data availability and time allows, these predictions will be extended to other chemical contaminants and stressors (i.e., pathogens) and other European regions. The outcome specifies the population fraction at risk due to interindividual variability in consumption and activity patterns and details the probability of this risk. The results allow policy-makers to take a better-informed decision on risks of human exposure to chemical contaminants through multiple environmental media and can have important implications for data gathering schemes on human consumption and activity patterns.

Derivation of a probabilistic NEC for mixtures (VU)

The subproject on derivation of a probabilistic NEC will start after month 6. Research will be focussed on modelling the effects on mixtures of 2 compounds, with survival and reproduction as

end points. This model activity includes a toxicokinetic module, a module for effects on (compound specific) physiological target parameters and a module for the translation of these effects on endpoints. Statistical methods will be developed to test the model against experimental data; these methods will be applied to data from the literature and the archives of the department, and to the first experimental data from RP3. Special attention will be given to a particular type of mixture: that of a molecular and the ionic form of the compound. The relative abundance of these forms depends on the pH, and so on the total concentration of the compound.

Harmonisation of analytical frameworks for meta-analysis of human and ecological toxicity data (DESUN)
A desk top study will be performed to identify options for harmonisation of the analytical frameworks used in the meta-analysis of human and ecological toxicity data. The results of the desk top study will be discussed by both partners involved in the derivation of probabilistic UFs (DESUN and USOUTH) and harmonisation will concentrate on using similar mechanistic descriptors for analysing the toxicity data, e.g., substance parameters, the genetic predisposition of receptors and the toxicological mode of action of substances.

Human and Interspecies uncertainty factors (USOUTH)
Most human polymorphic pathways for xenobiotic metabolism (CYP2C9, CYP2C19, CYP2D6, NAT) have been shown to be highly variable so that the current default kinetic uncertainty factor would not cover healthy adults for contaminants handled via these routes nor the most sensitive subgroups of the population (neonates and children; CYP2C19 and CYP2D6). During the first eighteen months of WP4.1, a meta-analysis of human pharmacokinetic data using data for markers of acute and chronic exposure in subgroups of the population (healthy adults, inter-ethnic differences, children and the elderly) will be performed for single compounds and chemical mixtures (drug interaction data) handled partially and totally via these polymorphic enzymes. This will provide new uncertainty factors (to cover the 95^{th}, 97.5^{th} and 99^{th} percentiles) taking into account the full extent of the variability for each polymorphic pathway and for each subgroup of the population. The human kinetic data will then be compared with the available animal data (mouse, rat, rabbit and dog including neonatal animals) to quantify interspecies differences and generate uncertainty factors for this aspect of uncertainty. Finally, a preliminary study of inter-individual and interspecies differences in pharmacodynamics will be performed to provide a basis for the derivation of uncertainty factors based on mechanistic data. These uncertainty factors can then be used by risk assessors and risk managers to replace the traditional uncertainty factors.

Comparative risk assessment (EPFL)
The Assessment of Mean Impacts method (AMI) adapts the traditional concept of ecotoxicological risk that is based on the most sensitive species, to a more discriminating and comparative assessment using the geometric mean response of species and its associated confidence interval. The method has proved to be a useful tool for the comparison of the potential hazard of substitutable pesticides on freshwater ecosystems (Humbert *et al*. 2003) with a validation of its environmental relevance (Fawer 2003). Based on the AMI method, a new ecotoxicological effect model was developed in the FP5 OMNIITOX project. The current aim is to integrate toxicity of mixtures and other stressors (e.g., eutrophication, acidification, etc) in this method. Firstly, the main stressors will be identified and selected for integration in the comparative assessment method. Secondly, the models quantifying the stressors will be analysed to reveal their compatibility with the comparative risk assessment approach. Finally, a methodological framework for comparative risk assessment will be developed including chemical mixtures, eutrophication, acidification and other stressors.

Deliverables

D.4.1.1 (month 18): Report on separation of true uncertainty and interindividual variability in predicted risks of the Dutch population from exposure to pesticides through all relevant environmental pathways.

D.4.1.2 (month 18): A paper on the model formulation for effects of a mixture of 2 compounds and a discussion of the application of the model to experimental data.

D.4.1.3 (month 18): Consolidated report describing the metabolism, pharmacokinetic and preliminary pharmacodynamic data in human subgroups of the population and tests species. Derivation of uncertainty factors for each subgroup of the population and test species for polymorphic elimination pathways after exposure to a single chemical or to chemical mixtures.

D.4.1.4 (month 18): Report describing method for comparative risk assessment of multiple stressors.

Milestones[17] and expected result

M.4.1.1 (month 10): Selection of relevant stressors in the environment for inclusion in comparative risk assessment

M.4.1.2 (month 12): Availability of data for separation of true uncertainty and interindividual variability in human exposure model and decision on the extent of the modelling effort

M.4.1.3 (month 14): Analyses of models quantifying the selected stressors for comparative risk assessment

M.4.1.4 (month 18): Uncertainty factors allowing for human variability and interspecies differences for polymorphic pathways after single and multiple chemical exposure

[17] Milestones are control points at which decisions are needed; for example concerning which of several technologies will be adopted as the basis for the next phase of the project.