Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	TNO
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: Models for exposure to highly toxic chemicals

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The aim of experiments within the framework of this appendix is to evaluate, design or use animal models to characterize response at (sub)lethal doses of chemicals, with specified triggers for intervention. These procedures will also be appropriate to develop forensic tools and identification of biomarkers of exposure, or determine the biological fate of the chemical to which the animal has been exposed. Eventually, this paradigm will lead to defined triggers to treat, that will depend on the expected time to intervention of the drug to be tested. The intervention could be aimed at that provided by eg first responders (including decontamination or not), first aid based on triage (initial assessment of severity leading to assignment to follow-up care), during transport to hospital or intensive care, and additionally follow-up evaluation and treatment on a longer term basis (see appendices 3.4.4.3 and 3.4.4.4). In this respect, the public setting for which the models are to be developed requires an adequate identification of intervention points that would be feasible in a mass casualty situation or for example in a follow-up situation. In a military setting, other operational circumstances exist, such as adequate training of personnel, and rapidly available countermeasures based on threat evaluations. Among others, the intervention point determines which animal model is feasible to address the efficacy of a certain drug. For example, each clinical phase after exposure should be separately considered for intervention, based on the hypothesis to be tested. Initial treatments could be aimed at improving survival, or lowering the burden on follow-up intensive care, whereas long term treatments are most likely aimed at preventing debilitating impairments affecting the quality of life.

The experimental approach generally is as follows:

1. <u>Selection of an animal model, including exposure route</u>

The main classes of agents to be covered in the project are:

(1) lung damaging agents, such as chlorine, phosgene, PFIB; and blister agents, such as sulfur mustard, lewisite and ethylene oxide;
(2) toxicants acting on the nervous system such as botulinum toxin, organophosphorus pesticides and nerve agents;
(3) cell toxic agents such as cyanides.
Among these classes of compounds, certain highly toxic agents are considered as potential chemical warfare agents.
A species will be selected, based on the (expected) human pathophysiology following exposure to the chemical agent (route, dose, timing; depending on the expected exposure levels of the public in a certain setting.
The exposure route will be determined based on the known toxicokinetics and – dynamics from the literature as available in the animal species selected. Exposure routes can be realistic in nature, such as inhalatory for volatile agents or aerosols, or for example dermal or oral for low volatiles. As a model however, it is also possible to control the toxicokinetics of an agent to mimic a certain exposure route using intravenous infusion, or to use subcutaneous administration. Consequently, the toxicological effects will follow a mechanistic pattern of toxicity similar to the modelled exposure route. Exposure can be achieved in anesthetized animals or in freely moving animals, depending on the desired outcomes for the animal model. The anesthesia to be used should not interfere with the mechanism of toxicity, or expected to mask or overestimate the efficacy of a treatment in follow-up stages of research, as described in appendices 3.4.4.3 and 3.4.4.4.

2. Preparation: Selection of readout parameters

Depending on the pathophysiology, a range of readout parameters will be selected, that should allow for defining possible intervention times (trigger to treat), and consequently allow for determination of therapeutic efficacy of such interventions in later stages in the project (appendices 3 and 4). The following list is a representation of possible readout parameters, that could be assessed over time, ranging from 2-3 hours to ~24-48 hours.

a) Clinical signs observation of the animal for specific signs such as chewing, salivation, involuntary movements, labored breathing, skin coloring

b) *Physiological signs*:
- brain activity (EEG), assessed using implanted telemetry devices
- cardiovascular parameters (ECG, blood pressure, heart rate), assessed using implanted telemetry devices
- respiratory parameters (respiratory rate, bronchoconstriction etc), assessed non-invasively using whole body plethysmography

c) *Blood parameters* – assessed in blood samples, obtained from unrestrained animals (pre-implanted indwelling catheter) or brief restraint (e.g. tail vein) in anesthetized or unanesthetized animals
   a. Toxicokinetics: levels of agent in blood
   b. Biochemistry: specific enzyme activity, representative for a specific chemical, specific chemical adducts (biomarkers)
   c. Clinical chemistry: Oxygen levels, ionic balance, enzyme/ protein levels

Primary indicators for progression of toxicity will be clinical signs. For agents primarily acting on the central nervous system, EEG will be included, whereas for lung damaging agents respiratory parameters are more indicative for progression of toxicity. Heart rate is a primary indicator for systemic burden of a poisoning, and can be an important parameter to assess the humane endpoint in case of lethal doses. Additionally, blood samples could be obtained to assess toxicokinetics when considered of additional value, and/ or to obtain biomarkers or general clinical chemistry. A designated combination of parameters should lead to a dose – time to effect range following exposure to a specific chemical.
In selected cases ultimately death, or time to death, or humane endpoint (See J) could be a readout. To establish a dose and time to effect relation, dose range finding, based on the literature, will be performed.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The animal procedures for experiments covered in this appendix can be divided into two phases, (1) a preparation phase in which animals can be equipped with cannulas, telemetrical devices according to the strategy described above. This is followed by a challenge phase (2) in which the same animals are exposed to a certain dose of a chemical agent and progression of the toxicity is monitored.

Phase 1: preparation

All surgical procedures will be carried out under species- and procedure appropriate anesthesia. These will be specified in the individual research plan. Appropriate peri-operative care will be provided (such as analgesia). Animals will be surgically equipped with one or more of the following:

Jugular vein and/ or carotid artery cannula (rats, guinea pigs, minipigs)

For permanent venous or arterial access (to draw blood or to inject intravenously), it is necessary to implant animals with an indwelling catheter in accessible blood vessels, such as the jugular vein or the carotid artery. The catheters will be exteriorized to allow for less invasive access. An example is subcutaneous tunneling, and fixation to the skull bone with dental cement in small animal models. In minipigs, temporary access via for example butterfly needles can be used.

EEG electrode (telemetry) (rats, guinea pigs, minipigs)

EEG electrodes to obtain brain electrical activity will be placed via the skull bone to access to the dura mater. The head of the anesthetized animal is fixed, and screw electrodes or flexible leads will be fixed to the skull. The electrodes are attached to a connector or telemetric body, and fixed with for example dental cement. Screws and instrumentation will be appropriately dimensioned for the animal species under study.

ECG Electrode (telemetry) (rats, guinea pigs, minipigs)

ECG leads are placed in an appropriate configuration, such as Lead II configuration, e.g. one lead at the right collar bone and one lead at the lower left chest. Leads are fixed in superficial muscular layers. The leads are led subcutaneously to a connector fixed on the skull (see EEG electrode), or to a telemetric body. In some cases the wireless transmitters are implanted in the animals instead of exteriorized as described above.

Skin microdialysis probe (rats, guinea pigs, minipigs)

A skin microdialysis probe (flexible) will be inserted in the skin of an animal, via a guide that is inserted over a fixed distance in the skin, e.g. 1 cm. The probe is fixed to the skin with glue or a suture (concentric probe).

Brain microdialysis guide (rats, guinea pigs)

Animals will be fixed in a stereotaxic frame under anesthesia. Via the stereotact, the guide will be aimed at a specified brain area by stereotaxic coordinates. Holes for screw fixation and probe guide access will be prepared using small electrical drills. The probe is fixed to the skull and fixation screw with dental cement.

Phase 2: Challenge phase

Phase 1 will be followed by a challenge phase as described below. In certain cases, it can be scientifically feasible to challenge animals while under anesthesia, which depends on the mechanism of toxicity (absence of interference with toxic agent). Additionally, the risk for workers performing the challenge will be considered.

Although intuitively a toxic challenge under anesthesia would be preferred from an ethical point of view, interference of the anesthesia with the mechanism of action will make the experiment obsolete, and accordingly unethical to perform. In such cases, the challenge will be performed in awake animals, after an appropriate recovery period of minimal 3 days up to 2 weeks, depending on the instrumentation and prechallenge surgery.

Animals can be exposed to agents via various routes, depending on the nature of the research question. Generally, gentle fixation by hand is used to restrain the animals. Depending on the research question, animals could be exposed under anesthesia. Animals can be exposed according to the routes, described below. Dose and route depend on the nature of the research question and will be in line with Diehl *et al*. Journal of Appl. Tox 2001; "A good practice…"

*__subcutaneous__ injection (all species)

*__intravenous__ injection

Under restraint via the tail vein (rat, mouse), under anesthesia in the penile vein (guinea pig) or via indwelling permanent catheter (all species), or temporary via butterfly access for minipig

*__intramuscular__ injection (all species)

*__percutaneous__ exposure (all species) (dropping a known amount of agent or agent solution on the skin; ul range)

*__inhalatory__ exposure (all species)

Animals will be restrained in a Battelle Tube (small animals) or a mask will be applied (minipig). The tube will be attached to the generation system, and the animal will be exposed nose-only for a predefined time

3 Monitoring (up to ~48 hours)

Blood sampling *(all species)*

Generally from the tail under restraint (rat, mouse: relatively small volumes with long intervals), via an indwelling cannula for guinea pigs or when larger volumes and short intervals are needed. Temporary vein access with e.g. butterfly needles will be applied in minipig). Maximum volumes will be determined according to Diehl *et al*. Journal of Appl. Tox 2001; "A good practice…"

Microdialysis sampling (rats, guinea pigs)

A probe will be inserted under gentle fixation in the brain guide ~24 hours before the experiment. Flexible tubing will be attached to the fixed probes and dialysates will be collected.

Metabolism (rats, guinea pigs, mice)

Animals will be individually housed in metabolism cages, with access to water and food *ad libitum* to allow for collection of faeces and/ or urine.

EEG, ECG monitoring (Telemetry) (all species)

The electrical signals (ECG/EEG) will be wirelessly transferred, from the wireless transmitter attached to the head stage (rats, guinea pigs). The animals will be housed individually during assessment to prevent interference of the signals of the different animals. Similar approaches can be used in minipigs, using equipment appropriate for minipigs (larger transmitters with increased signal range)

Respiration monitoring (rats, guinea pigs)

Animals will be placed individually in a whole body plethysmograph. In this way, breathing patterns can be assessed non-invasively. The animals will be in the plethysmograph for 24-36 hours, depending on the sampling time required. Water and food are available during measurements lasting more than 4 hours

At the end of the monitoring phase, tissues will be collected after appropriate euthanasia.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of animals in this phase, depending on the aim of the study should be sufficient to allow for determination of a time to effect baseline with sufficiently small deviations to increase power for efficacy experiments in treatment studies. Animals will be dosed in a staged fashion (unless there are scientific reasons to incorporate all animals simultaneously) i.e. once one/two animals are dosed, the outcome will be observed and if necessary the dose adjusted before continuing with additional animals. For example, if signs arise too fast, and lead to instant signs of poisoning or reaching humane endpoint outside the medical intervention to be tested, the dose will be lowered. On the other hand, if the challenge is too low, no signs exist and the anticipated treatment dose or time would not have to be administered. A typical experiment can be aimed at finding 1 dose (dose/ time to effect), to tailor the medical intervention, or can be aimed at determining a range of doses, such as subclinical, sublethal, or lethal, to find effects of a fixed treatment regimen.

Signs of poisoning can consist of clinical signs, cardiovascular compromise (blood pressure drop/ increase), onset or duration of seizures, respiratory effects (bronchoconstriction, hyperventilation), where a statistically significant increase or decrease can be defined as a toxicological effect. Mild or minimal effects might be considered subclinical (i.e. not substantially, or reversibly, influencing a critical parameter), whereas higher doses will induce sublethal or lethal effects, that should be mitigated.

Based on the results from this phase, the number of animals based on statistical power for treatment studies (appendix 3) can be properly assessed.
Additionally, in case blood levels of the chemicals are available, toxicodynamics will be evaluated. This will allow aiming for particular plasma levels of agents, in relation to the dose and future intervention.
In cases in which the aim is to find biomarkers of exposure (protein targets), the number of animals required per group may be lower, depending on the type of biomarker(4-6). For example, an *in vitro* identified target, will require verification at clinical doses *in vivo*, but lower variation is expected in such cases, or higher variation might be allowed, allowing lower numbers of animals. This can be for example the case in stages where only qualitative assessment is the case and not quantitative. This will depend on the sensitivity, selectivity and limit of detection of laboratory measurements (for example *in vivo* adduct verification using mass spectrometry based methods).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Based on the primary research question and available resources, an adequate animal model (species) will be selected. This can range from small animals (e.g. mice, rats, guinea pigs), to larger animals (e.g. minipigs) in later stages (see below).
With regard to development of challenge models of highly toxic compounds to test efficacy of medical countermeasures, initial research stages will have involved *in vitro*, *ex vivo* and *in vivo* studies. These studies are of value for insight into pathophysiology and feasibility studies, but they are insufficient to provide scientific evidence of efficacy and safety of medical interventions. Efficacy of a medical countermeasure in case of an exposure should meet at least 4 criteria:

- The pathophysiological mechanism and the reduction by the countermeasure should be reasonably well-understood (construct validity; in addition to literature pilot studies, in general *in vitro* data indicating the primary toxicological target will be available);
- The effect is demonstrated in more than one species with a predictive response (predictive validity);
- The endpoint clearly is related to the benefit in humans (face validity); and
- The expected and desired outcome of the medical countermeasure should be defined.

To this end, appropriate animal models have to be available. With respect to the animal model development, the range of models that could be used is quite broad, depending on the requirements, e.g. challenge agent, route of exposure, time-frame of exposure, human target and the desired benefit of prospective countermeasures on read out parameters (for follow-up research described in appendices 3.4.4.3 and 3.4.4.4). A general algorithm for selection is as follows:

a. Preselect a long-list of animal models from historic evidence.
b. On the basis of the challenge agent of the particular Task: consider the pathophysiology, the target organ(s) / disease patterns; take into account possible differences in the target population (such as gender, age, predisposition).
c. Create match between animal models considered appropriate for those target organs and target end points to be mitigated; the result is a re-ranked short-list.
d. On the basis of the drugs to be studied as potential countermeasures, for example based on translational PK/PD: match these with short-list (point c); the result is an updated short-list.
e. Starting off with list (d): take into consideration the route of exposure of the challenge agent (most likely inhalatory or dermally) and that of the drugs (most likely parenteral, oral or dermal) check for best fit with (operational) requirement.
f. Integrate all previous points to select appropriate models

This algorithm is in agreement with points addressed in the "Guidance for Industry: Product Development under the Animal Rule" (see Table in Project Proposal, paragraph 3.2) providing an excellent starting point for down selection of animal models for separate challenge agents and efficacy requirements for treatment guidelines. As the models in this appendix will be used for evaluation of therapeutic safety (appendix 3.4.4.2) and efficacy (appendices 3.4.4.3 and 3.4.4.4) at later stages, this has to be taken into account here already.

A extract of the schematic overview representing important factors for translational factors is shown in figure 1:

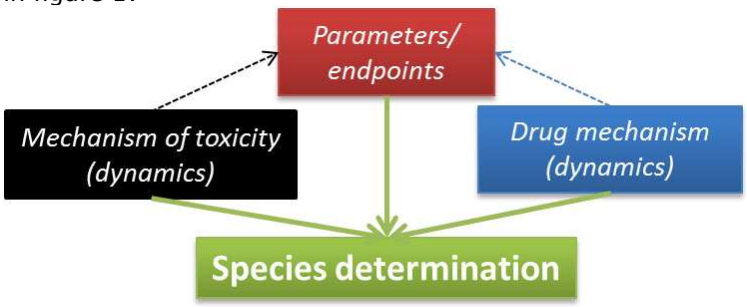

Figure 1. Overview of interplay between aspects for species determination.

The figure shows that translation from human studies to the target human population will be based on drug or agent plasma levels (so not dose!) that induce effectivity or toxicity in the animal models (pharmaco- and toxicodynamics). In case such information can't be obtained in actual human studies, such estimations will be based on in vitro ADME parameter translation. Additionally, efficacy should eventually be obtained at the plasma level of a drug that does not induce toxicity by itself, worse than the medical condition that has to be treated.

An overview of certain rationales with regard to read-out parameters or etiology are described below. As small animal models, rats, mice and (hairless) guinea pigs are appropriate models to address different research questions.

1. Rats are animals suitable for addressing research questions with regard to animal behavior or issues related to the central nervous system (CNS), including brain drug or agent levels and seizure development and culmination. This can be relevant for follow up questions with regard to improvements with regard to quality of life at long term, as specified in appendix 3.4.4.4.
2. (Hairless) guinea pigs are generally used to assess the effects of interventions (e.g. decontamination or treatment) on skin penetration and kinetics. To this end, the species with skin properties most closely approximating the human skin will be used. In addition to hairless guinea pigs, hairless rats have become available recently.
3. Mice can be used for certain distribution experiments, behavior or simple efficacy experiments whenever appropriate. In particular the broad availability of genetically modified mice can be of value to investigate fundamental mechanisms. The use of Genetically Modified Organisms (GMOs) can be of particular interest in case specific receptors or proteins are suspect of high contribution to toxicity of the compound or when those are serving as a therapeutic target. The choice of animal model depends on the specific gene(s) that have been inserted or deleted. An example model, that is however not yet fully established, is a Carboxylesterase knock-out mouse, or human Acetylcholinesterase knock-in model. A drawback of such a mouse model is that the possibilities to obtain physiological parameters or sufficiently large blood samples is lower, requiring larger number of animals.

In spite of a different airway architecture from humans, these small animal species can also be valid to elucidate mechanisms of toxicity of inhalatory agents, as in general the organ toxicity depends on the amount of agent reaching the target site, that should be similar to that in human. This can be titrated depending on the species.

At later stages, large animal models are used. This is mostly the last stage that is required as a replacement for phase III clinical trials. Large animal models accepted in this phase are non-human primates, and (mini)pigs. We only have ability to use minipigs. Thus, only if the minipig is valid for the toxicity and drug target (similar to that in humans, with a similar response, based on literature and based on selection as described above), read out parameters, such a model will be pursued. With regard to the minipig, information in the public domain as well as publications from the European Medicines Agency (EMA) and the Federal Drug Administration (FDA) shows that it is fully recognized and accepted

by regulatory authorities worldwide as a large animal model for humans, especially with regard to skin, cardiovascular system and respiratory system. The species has a well-documented genetic background, housing and feeding conditions and microbiological status. Background data with respect to growth curves/data, organ weight, hematology, clinical chemistry, hemodynamics and histopathology are well-known. In addition, as an objective after animal model development can be to prepare technology transfer, laboratories worldwide are equipped to handle minipigs.

Depending on the target population for registration of new medical treatments, juvenile, adult or aged animals will be used.
The choice of gender will be based on literature, and depending on the target population for drug use. Initial phases will most likely be performed in one gender only. From the animal model selection, it will be derived whether the response to toxic chemicals is gender specific or not. For the anticipated levels of exposure, to be investigated in the project proposal, the usually rather subtle gender related differences following exposure are not expected to be a pivotal factor, that could affect the outcome of medical countermeasures. However, to minimize possible variations, and as the discomfort induced by the experiments can be classified up to severe, initial studies will preferably be performed in one gender at a time, and in follow-up stages critical challenges and doses could be on request of regulation or scientific validation transferred to the opposite gender. A similar strategy is employed for age specific differences.
In case of absence of historical evidence, the choice for the single gender- and age category to be tested may also be influenced by the availability of animals at the supplier. For instance, if there is a surplus of adult female minipigs at the supplier and no significant gender- or age-specific effects are to be anticipated, adult females will be chosen for the study.

Estimated numbers in 5-year project period:

Annex I	Mice	Rats	Guinea pigs	Minipig
Total number of animals	60	200	200	80
Nr of studies	1-2	6-8	6-8	2-3
1 Terminal	60%	10%	10%	50%
2 Mild	0%	0%	0%	0%
3 Moderate	40%	50%	50%	30%
4 Severe	0%	40%	40%	20%

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement
The research described here will be follow-up research, from for example *in vitro* target identification. Signs of toxicity *in vivo*, are in most cases the result of cascade interactions in a body. For example, nerve agent poisoning highly selectively inhibits acetylcholinesterase, but the subsequent signs of toxicity arise from eventual recruitment of glutamatergic systems. Cyanide selectively interferes with intracellular oxygen metabolism, but this results in a number of changes in clinical chemistry, and cardiovascular compromise. As certain chemicals are highly reactive, the bonding to a key physiological target can be extremely strong (covalent binding), which requires an extremely targeted, highly reactive drug action. This is extremely challenging and in a number of cases chemically impossible (this requires *in vitro* research in critical organ cells for example, or just protein targets). It can be possible however, to intervene with the cascade effects, and let the body resolve the intoxication, by regeneration of targets for example. To achieve that, artificial respiration, or certain anesthetic regimens may be employed. Such cascade interactions cannot be replaced by *in vitro* approaches.
In general, for drug approval, phase III (Clinical efficacy) trials are necessary to indicate a medical intervention for a specific condition. As patient populations are not available for exposure to highly toxic chemicals, this phase is replaced by the testing in a qualified animal model under quality standards, which are currently not available.
Reduction
The number of animals used is reduced by well-designed studies (e.g. choice of dose, choice of dosing route, choice of animal, etc.). With regard to development of challenge models of highly toxic compounds to test efficacy of medical countermeasures, initial research stages will have involved *in vitro*, *ex vivo* and *in vivo* studies. In the proposed project, animals could for example be equipped with telemetric devices, in order to obtain a range of precise and adequate readout parameters. Known baseline variations from experience or other studies of critical parameters and possibly the variation in response to a chemical challenge will allow statistical power analysis to calculate the number of animals for a dose range finding experiment. Furthermore, the research strategy, up- and down dosing, described under A(2) allows the reduction of the number of animals per group. This will be based on sequential analysis, meaning that in case exposure of additional animals in a group will not lead to significant improvement or decline (the difference already being too big), the group will be terminated.
Refinement
See next question

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Pre-challenge surgery will always be performed under anaesthesia and appropriate peri-operative care including analgesia. Animals are housed according to EU regulations and provided appropriate cage space, bedding material and species specific environmental enrichment. Whenever scientifically sound, the animals will be kept under anaesthesia during the entire procedure. Furthermore, research designs will be optimized to obtain as much information as (technically) possible, taking into account possible increasing discomfort. Obtaining physiological data by telemetry requires one surgery, but after that less handling and discomfort is required. Similarly, placement of a cannula induces discomfort once, but does prevent repeated skin puncturing or restraint.
If necessary, for any animal showing signs of discomfort, measures will be taken to relieve this discomfort and, if necessary, treatment of the animals will be altered. If an animal shows signs of severe discomfort (humane endpoints), they will be humanely killed after consultation of the animal technicians, responsible researcher and/ or a designated veterinarian. In cases where (time to) death is a readout parameter, the duration of such signs will be kept as short as possible (~4 hours, in most studies), or in selected cases up to 24 hours. Survivors will be euthanized at a predefined time point within this time frame.
Studies will be performed in contained facilities specifically equipped for handling highly toxic chemicals

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Before any intervention, animals will be housed according to the guidelines, or also whenever possible.
In cases in which animals are instrumented by surgery, animals will be housed individually to prevent damage to cannulas or electrodes.
In case of individual housing, social interaction is limitedly possible, due to the use of open cages which allow sniffing and reaching out to neighboring cages. Appropriate cage enrichment will be available for long term housing, such as wooden chewing blocks, paper rolls, play houses, shelters etc., always taking into account that the animals cannot hurt themselves.
Some experimental housing situations (e.g. metabolic cages) warrant individual housing without bedding or enrichment. Period of housing in these situations are kept as short as possible (max ~36 hrs)

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Surgical procedures will be carried out under species appropriate anesthesia, such as isoflurane (4-5% induction, 1-2% maintenance) anesthesia, depending on surgical procedure and animal species. Specific anesthesia and analgesia this will be specified in the individual research plan.
Appropriate peri-operative care will be provided with analgesics and/ or antibiotics up to 48 hours.
Animals will be monitored frequently immediately after surgery until full recovery, based on voluntary movement and consciousness, then lowered to twice daily, or once at later stage (after ~1 week). See humane endpoints for observation criteria.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

The expected adverse effects could be laboured breathing, loss of consciousness, tremor, epileptic seizures, apnoea, cardiovascular compromise, all direct results from the toxic challenge.
In the unlikely event a surgery would be not successful, this could lead to compromised recovery. Measures to be taken in such a case are described in paragraph (J).

Explain why these effects may emerge.

The effects described are the direct result from a toxic challenge. The observation of these effects is the aim of the study.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

If scientifically valid, the exposure to toxic chemicals will be performed under anaesthesia. Surgery will be performed using appropriate analgesia and anaesthesia, and animals will be monitored closely for adverse signs during recovery from a procedure. In general chemical challenges will be as low as possible, without compromising the scientific aim. Furthermore, animals will be observed closely after a chemical challenge, and humane endpoints are defined. Depending on the severity, and aim, the experiments will be as short as possible to minimize the duration of discomfort (initially 4-6 hours, up to a maximum of 24 hours in this phase, depending on the aim of the model use).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

At high doses, the challenge will be lethal within a very short time (minutes to hours), in most cases initiated by rapid loss of consciousness. Animals might be challenged to a lower but toxic dose, leading to a longer period of lower severe clinical signs. In particular in those cases, a defined humane endpoint will be chosen, to minimize suffering, and additionally to obtain scientifically comparable outputs. A humane end point could be a certain duration of a decline in cardiovascular parameters, or breathing. In case spontaneous death resulting from the chemical challenge can be prevented (reaching Humane End point), the animals will be sacrificed.
In earlier phases, a failure from the animals to recover from surgery, i.e. has not returned to initial body weight at the day of exposure after surgery, might be an indication for exclusion from the experiment. A normal short body weight dip of around 10% can occur, but normally the animals rapidly return to their pre-operative weight within a 1-5 day recovery period.
Furthermore a drop in heart rate under ~35% of baseline level is an indication for early termination of the experiment. Specific end points will be specified per individual study plan.
Scoring for overall condition after surgery and chemical challenge:
0: Good (no clinical signs, normal weight gain, smooth fur)
1: Adequate (normal effects of low invasive surgery, normal feeding and drinking, stable weight, no weight gain)
2: Average (average signs of low invasive surgery, slightly decreased eating and drinking, bad appearance of fur, slight weight decrease (<20%), recovering weight)
3: Poor (bad appearance of fur, weight decrease >20%, lethargy, poor coordination, no eating and drinking)
4: Bad (no eating, drinking, weight decrease >30%, progressive weight loss >2 days, Painful, hunched posture, or worse)

Conditions 0-2 are expected to recover, progression to 3 could occur for a maximum of 1 day, but should return to 2 the next day. Progression to score 4, or score 3 for 2 days indicates a very bad recovery, and will indicate removal of the animal of the study and euthanasia in consultation with the Animal Welfare Body.

Indicate the likely incidence.

In the majority of cases, at lethal and sublethal challenges, the animals will reach the humane end point, or lose consciousness rapidly (within minutes to hours after exposure to the toxic agent), as an endpoint of the study.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Annex I		Species				Discomfort			
	Procedure	Mice	Rats	Guinea pigs	Minipig	1 Non-recovery	2 Mild	3 Moderate	4 Severe
Preparation	Cannulation		x	x	x			x	
	Butterfly access				x		x		
	EEG electrode placement		x	x	x			x	
	ECG electrode placement		x	x	x			x	
	Skin microdialysis probe placement		x	x	x			x	
	Brain Microdialysis guide placement		x	x				x	
Challenge	subcutaneous injection	x	x	x			x		
	intravenous injection	x	x	x	x		x		
	intraperitioneal injection	x	x	x			x		
	intramuscular injection		x	x	x		x		
	dermal application (percutaneous)		x	x	x		x		
	Inhalation	x	x	x	x		x		
Monitoring	Anesthetized model		x	x	x	x			
	Blood sampling	x	x	x	x		x		
	Telemetry	x	x	x	x		x		
	Subclinical	x	x	x	x		x		
	Sublethal	x	x	x	x			x	x+
	Lethal	x	x	x	x			x	x+
	Euthanasia	x	x	x	x	x			

non-recovery: for challenge under anaesthesia immediately after instrumentation
moderate : for short term acute challenges, where loss of consciousness appears within ~60 minutes after onset of moderate clinical signs. Within the scientific boundaries of the project, rapid death might be an outcome parameter. It is recognized that this intuitively could be classified as "severe" discomfort. However, the anticipated speed of the progression of toxicity, and in most cases rapid loss of consciousness in case of challenging awake animals, eventual discomfort could be rated as "moderate".
+: Severe: In case of lower dose challenges, the effects arise more slowly and will be of longer duration. Such effects would be severe bronchoconstriction, or for example paralysis. This can lead to a maximum of 'severe' discomfort, when a longer duration of observation is required for the model (> 8 hours, up to

24 hours). However in that case the HEP will be taken into account, and the duration of effects will be kept as short as possible (~4-6 hours). If HEP can be applied in an early stage, the discomfort of the animals could also be classified as "moderate".

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Tissues are required for analysis of organ toxicity, or for example amounts of chemical agent in the tissues. This will be at a fixed time point following challenge, or after reaching HEP.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes