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SCIENTIFIC OPINION

Scientific Opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile[1]

EFSA Panel on Plant Protection Products and their Residues (PPR)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

This is an updated version of the Scientific Opinion published on 12 July 2013 which was amended after a public consultation that ran from 17 July to 30 September 2013. The outcome of the public consultation is available at http://www.efsa.europa.eu/en/efsajournal/doc/538e.pdf[4].

ABSTRACT

The European Food Safety Authority asked the Panel on Plant Protection Products and their Residues to develop an Opinion on the identification of pesticides to be included in cumulative assessment groups (CAGs) on the basis of their toxicological profile. In 2008, the PPR Panel adopted an Opinion on the suitability of existing methodologies for cumulative risk assessment of pesticides and a tiered approach was proposed, which was applied to a selected group of triazole pesticides in 2009. The present Opinion suggests a methodology for grouping of pesticides based on phenomenological effects and provides CAGs for the thyroid and nervous system. This approach can be applied even when the underlying biochemical events mediating the effects are not understood, and is based on a standardised and thorough review of Draft Assessment Reports (DARs) supporting the approval of all pesticides in Europe, and on recommendations from the European Commission. Pesticidal active substances exhibiting neurotoxic properties were allocated to CAGs for acute effects on motor, sensory and autonomic divisions of the nervous system and neurochemical endpoints. Chronic effects across the same divisions/endpoints and neuropathological effects were collated. Active substances having adverse effects on the

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[4] The main changes from the original opinion are as follows: (1) data collection spread-sheets: some NOAELs/LOAELs were replaced, clarifications were added to the remarks column, and a number of substances missing from the original data collection were added; (2) the cumulative assessment groups for both the nervous and the thyroid systems have been updated in the text; (3) the lists of substances that were assessed and selected for cumulative assessment groups (Appendix H) have been updated; (4) the paragraph on developmental neurotoxicity has been updated according to the new data requirements for active substances used in plant protection products in Regulation (EU) No 283/2013. The original opinion and spread-sheets are available on request as are copies showing all the changes that were made.

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thyroid system were allocated to CAGs for effects either on C-cells/the calcitonin system or on follicular cells/the T3/T4 system. The PPR Panel notes that the resulting groups encompass many pesticides and also that individual pesticides could appear in several groups and therefore the data entries for performing cumulative risk assessment (CRA) are of considerable magnitude. Although some CAGs contain a large number of pesticides, little indication of cumulative risk may be inferred from the size of CAGs per se. The PPR Panel recommends that the methodology is implemented for all major organ/systems but the approach used should be considered specific for pesticides.

KEY WORDS

Cumulative risk assessment, toxicological profile, methodology, cumulative assessment grouping, thyroid, nervous system

SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Plant Protection Products and their Residues (PPR) to deliver a scientific Opinion on the identification of pesticides to be included in cumulative assessment groups (CAGs) based on their toxicological profile, the aim being to develop cumulative risk assessment (CRA) methodology.

This Opinion was preceded by two previous Opinions (EFSA, 2008, 2009). In the first one, the PPR Panel evaluated existing methodologies on cumulative risk assessment (CRA), and recommended that a tiered approach should be adopted both for hazard and exposure assessments. Criteria for grouping active substances into CAGs were proposed, based on the chemical structure, mechanism of pesticidal action, mode/mechanism of mammalian toxicity and common toxic effects. In the second Opinion an exercise was carried out to test the proposed approach by a worked example of a group of triazole pesticides, a well-defined group in terms of structure, pesticidal mode of action and toxicological effects (EFSA, 2009). Thus, the previous opinions dealt with CRA, encompassing both hazard and exposure assessment. In the Terms of Reference of the current opinion, EFSA has requested for a scientific Opinion on the identification of pesticides to be included in CAGs on the basis of their toxicological profile and deals therefore solely with hazard assessment. The Panel is aware that the conduct of CRA is a process that involves several steps and multiple considerations, many of which go beyond the scope and Terms of Reference of this opinion. CRA has to include the outcome of the current Opinion as well as other critical elements, such as the availability of occurrence data and the scientific and technical capacity of exposure assessment methodologies. Recommendations on the conduct of CRA were outside the scope of the present opinion.

The present Opinion presents a general methodology and criteria specifically developed for establishment of CAGs for pesticides. The methodology has been applied to establish CAGs for pesticides having effects on the thyroid and nervous system, and has been developed on the basis of datasets of oral toxicity studies evaluated in draft assessment reports (DARs). The methodology was developed in order to take cumulative effects into account in the decision on applications concerning maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin. The CAGs derived from this methodology could in principle be used to support CRA resulting from non-dietary exposures (i.e. operator, worker, bystander and resident exposure).

The allocation of pesticide active substances into CAGs requires a standardised and thorough review of the DARs for effects on individual organs and organ systems of all approved pesticides relevant for dietary exposure. Therefore, two preparatory projects for collecting toxicological data from pesticides were initiated. In the first project, all pesticides authorised prior to 31st of May 2009 were evaluated. The contractors proposed a grouping approach starting from identifying toxicological target organs and organ systems and then subsequently refining the grouping by identifying a specific phenomenological effect. If data allowed, the grouping was further refined by identifying a common mode or mechanism of action. The data collection and approach proposed by the contractor was scrutinised and partly consolidated by the PPR Working Group. It was decided that the data collection needed to be re-evaluated and, hence, a second project was launched specifically consolidating identified pesticides having effects on the nervous system, the liver and the reproductive and developmental system. In addition, pesticides approved from 31st of May 2009 until 1st of January 2012 were included in the scope of the second project.

The PPR Panel acknowledges that EU residue monitoring programmes indicate that there is some consumer exposure to residues of non-approved pesticides which should also be included in CAGs.

Following the work undertaken by the PPR Working Group for the current Opinion on reviewing pesticides for inclusion in various CAGs, it became apparent that there are often few or no data available on mode of action, but that many compounds affect the same target organ and/or cell population. On this basis, the proposed methodology follows a phenomenological approach based on organ or system toxicity, consisting in including in a CAG for a specific effect all pesticides causing

this effect, even if the underlying mode of action (MoA) is unknown. Interactions (synergisms or antagonisms) are not expected to occur at the low exposure levels of residues that are observed in monitoring programs. Thus, the PPR Panel considers that mainly dose additive effects of substances are normally relevant to CAGs that may be used in the context of MRL setting (EFSA, 2008; Boobis et a., 2008).

As there may be limited opportunity for refinement of CAGs on the basis of available information on mode/mechanism of action, the proposed grouping methodology makes a sufficient precautionary approach, which is agreed upon by the European Commission and EFSA: when insufficient or no information is available, it is assumed that chemicals with the same effects may have a similar mode of action, even though they exhibit a wide range of chemical structural features. This view is based on empirical evidence that chemically unrelated substances may have a common effect in target organs/organ systems, which can be well approximated by dose addition (Kortenkamp et al., 2009). This has to be considered within the context of pesticide evaluations by EFSA and hence the approach recommended in the present Opinion differs from the approach tentatively used by the PPR Panel in its previous work.

The stepwise methodology for grouping has been elaborated to address acute and chronic dietary efects.

The methodology comprises four main steps as follows:

- Identification of the specific effects by:

  i) exclusion of local effects
  ii) exclusion of non-adverse effects
  iii) exclusion of effects not relevant to humans
  iv) evaluation of the unambiguous nature of the effect
  v) identification of non-specific effects

- Characterisation of the specific effects
- Data collection
- Grouping of pesticides into CAGs

The PPR Panel recommends that the implementation of the methodology based on specific effects should be supported by expert judgement in order to identify the effects relevant for grouping according to the criteria laid down in the opinion. In particular, expert judgement is required to identify and characterise substances that can trigger different outcomes of the same toxicity pathway (e.g. different effects on motor division of the nervous system) or that may cause toxic effects at multiple sites by a single mode of action (e.g. acetylcholinesterase inhibition).

The CAG methodology in the current Opinion has been applied to the nervous system and the thyroid system.

For the identification and characterisation of the potential neurotoxicity of pesticide active substances, the functional divisions of the nervous system (motor, sensory and autonomic) along with the cognitive domain, neurochemistry and neuropathology parameters were considered as potential targets. Indicators of specific neurotoxic effects were identified and applied to characterise the CAGs for the nervous system.

A total of 68 active substances, were identified as having specific effects on the nervous system. Additional four substances were excluded from grouping because the methodological criteria were not met and/or the exposure to these substances by the oral route was highly unlikely following their authorised use.

The CAGs of substances identified as neurotoxic are presented in two separate tables for acute and chronic effects, respectively. Data were tabulated according to the level of organisation of the nervous system, the indicator of the specific neurotoxic effect, the active substance, its mode of action and the lowest NOAELs and/or LOAELs for each indicator. Non-specific or secondary effects, as well as effects that occur after administration of high doses, resulting in severe systemic toxicity, were not included in these CAGs according to the criteria for identification of specific effects listed above.

The following groups were proposed (number of pesticides in each group):

- Acute exposure (47)
  - Motor division (45)
  - Sensory division (20)
  - Autonomic division (29)

- Chronic exposure (64)
  - Motor division (53)
  - Sensory division (21)
  - Autonomic division (24)
  - Neuropathological changes (19)

The Panel recognises that the neurochemical parameters, i.e. brain or erythrocyte AChE inhibition, represent a level of grouping for neurotoxic substances based on mechanism of action rather than on phenomenological effect. However, AChE inhibitors play a prominent role in the risk assessment that would result in an increased sensitivity for some substances. For this reason, and to keep consistency in the grouping approach, the neurochemical parameters should be used for further refinement when this mechanism of action is recognised. In addition, neuropathological changes were considered relevant only for chronic CAGs since some pesticide active substances induced morphological changes as the only adverse effect or they were found to be the most sensitive ones.

Despite the effects of pesticides on the cognitive domain e.g. learning and memory, which are relevant for assessment of neurotoxicity, the information available in the DARs failed to identify these effects. This is very likely because these effects correspond to a higher tier of assessment that was not performed on a routine basis during the toxicological assessment of pesticides.

Owing to the absence of systematic testing of pesticides for Developmental Neurotoxicity (DNT) in the European Union, and in consideration that new data requirements for active substances used in plant protection products have just been recently introduced (Regulation (EU) No 283/2013 of 1 March 2013)[5], results from such tests, even when in certain instances available (e.g. for dimethoate, fenamiphos, fipronil, malathion and molinate), have not been considered for CAGs in the present opinion. Since the thyroid functions as a gland that produces systemically acting hormones (calcitonin, thyroxin (T4) and triiodothyronine (T3)), the most conservative level of grouping (CAG 1) was defined by effects occurring on the organ (thyroid) or organ system (hypothalamic-pituitary-thyroid axis), e.g. through changes in thyroid hormone levels (in total 101 of 287 screened substances were identified as affecting the thyroid or thyroid hormone systems). Identification of specific effects concerning two different thyroidal cell populations/hormone systems formed the basis for further refinement, yielding two sub-groups at the second level (CAG2A and CAG2B).

Substances affecting C-cells of the calcitonin system were allocated to CAG2A (22 substances). Owing to interrelationship of the specific effects between C-cell hyperplasia and neoplasms, and

[5] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal L 93, 1-84. 3 April 2013

absence of information on underlying mechanisms, further sub-grouping of thyroid CAG2A was not possible.

Substances affecting the thyroid follicular cells and the T3/T4 system, i.e. displaying changes in circulating T3/T4 or TSH levels, follicular cell hypertrophy/hyperplasia or follicular cell neoplasia, were allocated to CAG2B (in total 96 substances). The specific effects that were used to define the CAG2B sub-group were apparently interrelated or connected to one another by a chain of events. While the precise mechanism of action is currently unknown for many substances within CAG2B, several different mechanisms of action are expected to contribute to a final deleterious common effect (i.e. decrease in T3/T4 action). For these reasons and based on the information available in DARs, further refinement of grouping is currently not possible. In exceptional cases, where there is convincing evidence for substance-dependent direct stimulation of the thyroid or hyperthyroidism, exclusion of substances from this sub-group might be considered.

The application of grouping methodology has yielded CAGs with sometimes large numbers of pesticides.The Panel notes that although some CAGs contain a large number of pesticides, little indication of cumulative risk may be inferred from the size of CAGs per se. The Panel further notes that, even within large CAGs, the majority of pesticides might not contribute significantly to a given combination effect, either because exposure is very low, and/or because potency in relation to the effect considered is weak. Instead, cumulative effects are likely to be driven mainly by a few active substances within the group.

Comprehensive preliminary work has been done on effects on the liver, adrenals, eye and developmental and reproductive system and provides a starting point for developing CAGs also for these systems in the future.

The PPR Panel identified a number of uncertainties and limitations in grouping of pesticides according to a common or shared toxic effect. In particular, a grouping based on toxic effects rather than on mode of action will lead to more uncertainties in predicting possible combination effects. However, the Panel acknowledges that when limiting CAGs to known common mode of action, thereby excluding pesticides for which information on mode of action is not available to enable their inclusion in relevant CAGs, the degree of uncertainty in CRA would also increase. Thus, a higher level of protection can be afforded by considering a wider range of pesticides and until information on precise modes of action becomes available, the cost of this is to use an effect-based approach that introduces some uncertainties around combination effects. Additional uncertainties considered by the Panel included the levels of details of the toxicological assessments in the DARs, changes occurring over the years in regard to data requirements and study protocols of the toxicological assessments, and inconsistency and variability in terminology of the DARs.

The PPR Panel also makes recommendations for the implementation of CAG grouping methodology in CRA to support MRL setting. The PPR Panel also notes that further refinement of grouping maybe achieved when data on the precise toxicological mode of action are available. However, information that justifies any deviation from dose-addition might also be necessary to consider for such a refinement. In addition, non-approved pesticides detected in food commodities should be included in CAGs, and a sound and consistent procedure for data retrieval should be developed for both the methodology and the inclusion of new substances into the relevant groups.

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BACKGROUND AS PROVIDED BY EFSA

Regulation of the European Council and the European Parliament (EC) No. 396/2005[6] on Maximum Residue Levels (MRLs) emphasizes the importance "of carrying out further work to develop a methodology to take into account cumulative and synergistic effects of pesticides" as there is currently no *internationally agreed* methodology available for these purposes.

On 28/29 November 2006, EFSA started working on CRA of pesticides by organising a colloquium on "Cumulative risk assessment of pesticides to human health: the way forward". The summary report of this colloquium is published on EFSA's website:

http://www.efsa.europa.eu/en/science/colloquium_series/colloquium_7.html

This report includes the results from two discussion groups dealing with cumulative exposure.

In addition to this colloquium organised by EFSA, WHO/IPCS hosted an International Workshop on Aggregate/Cumulative Risk Assessment in Washington in March 2007. The report can be found on: http://www.who.int/ipcs/en

Based on the results from these international events, EFSA's Scientific Panel on Plant Protection Products and their Residues (PPR Panel) elaborated an Opinion "to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risk from pesticides to human health with a view to set MRLs for those pesticides in the frame of Regulation (EC) No. 396/2005".

In this Opinion the PPR Panel proposed criteria required to be met for inclusion of compounds in a cumulative assessment group (CAG). It highlighted the possibility of using different levels of refinement in a step-wise approach. The grouping can be based on general criteria like chemical structure, or mechanism of pesticidal action, or higher level criteria like common toxic effect, common phenomenological effect or even toxic mode of action.

Following this opinion, a worked example of the proposed methodology was developed for a group of triazole compounds and the results are reported in a separate Opinion with suggested refinements as necessary. This Opinion of the PPR Panel was adopted in June 2009.

As a third step the PPR Panel is providing assistance to the evaluators and regulators elaborating an Opinion containing lists of pesticides included in cumulative assessment groups on the basis of their toxicological characteristics. In the preparatory phase of this Opinion the PPR Panel has launched a call for proposals CFP/EFSA/PPR/2009/01 based on article 36 of European Parliament and Council Regulation (EC) No. 178/2002[7]. The aim of this project is to set the basis for carrying out CRA routinely in the context of MRL regulation by searching for and exploring the existing pesticide data bases, open literature and Draft Assessment Reports (DARs) to identify the toxicological effects and their indicators that can be used for CRA. Proposals for cumulative assessment groups of active substances causing these identified effects and related indicators (including the selection of index compounds) are to be made in the final report of this project.

This report will be the starting document for the scientific Opinion of the PPR Panel on identification of cumulative assessment groups.

[6] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Official Journal L 70, 1-16. 16 March 2005.

[7] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal L 31, 1-24. 1 February 2002.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The Scientific Panel on Plant Protection Products and their Residues is asked by EFSA to prepare a Scientific Opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile.

ASSESSMENT

1. Interpretation of the Terms of Reference by the PPR Panel

In the Terms of Reference EFSA has requested for a scientific Opinion on the identification of pesticides to be included in cumulative assessment groups (CAGs) on the basis of their toxicological profile.

In the explanatory background provided by EFSA, reference is made to the Regulation (EC) No 396/2005 of the European Council and the European Parliament on Maximum Residue Levels (MRLs), which lays down in article 14 that, in decisions on applications concerning MRLs, *account shall be taken of the possible presence of pesticide residues arising from sources other than current plant protection uses of active substances, and their known cumulative and synergistic effects, when the methods to assess such effects are available*. As the regulation stipulates that only pesticides need to be considered for cumulative and synergistic effects, the Panel restricted its Opinion to CAGs of pesticides with a view of supporting specifically the regulatory MRL-setting process of pesticides in the European Union. The Terms of Reference state that the CAGs should be based on the toxicological profile of pesticides and not on exposure considerations. The PPR Panel will therefore only use toxicological considerations for recommendations regarding CAGs of pesticides. Accordingly, the current Opinion does not consider the assessment of cumulative effects of chemicals other than pesticides; the opinion's outcomes might, however, contribute to the on-going elaboration of a possible all-embracing methodology for the assessment of cumulative risks from all types of chemicals and from all sources of exposure.

The Panel recognises that the conduct of CRA is a process that involves several steps and multiple considerations, many of which go beyond the scope and Terms of Reference of this opinion. CRA is therefore an exercise which has to consider the outcome of this opinion, as well as other critical elements, such as the availability of occurrence data and the scientific and technical capacity of exposure assessment methodologies. In this Opinion the PPR Panel does not make any recommendations for the conduct of CRA in a general sense.

To ensure coherence between risk assessment and risk management in dealing with the request of the current opinion, EFSA has in addition formally consulted the European Commission on general recommendations regarding the desired level of protection and has informed the Panel about the outcome of this consultation.

2. Introduction

The European Food Safety Authority (EFSA) asked the Panel on Plant Protection Products and their Residues (PPR) to provide a scientific Opinion on the identification of pesticides to be included in cumulative assessment groups (CAGs) based on their toxicological profile to develop CRA methodology.

A key step in CRA is to decide which substances should be grouped together in CAGs for an assessment of their combined effects, and which criteria should be applied in defining such CAGs. The PPR Panel has already adopted two opinions on CRA that deal with this issue.

In the first of these opinions (EFSA, 2008), the Panel highlighted the possibility of different levels of refinement for defining CAGs in a step-wise approach. It was proposed that groupings can be based on general criteria including the chemical structures of active substances, mechanism of pesticidal action, or more refined criteria such as shared toxic effects, or toxic modes of action. In addition, the Panel identified more specific criteria for selecting CAGs for consideration in CRA. These include: (a) frequency of detection in monitoring programmes, (b) high use based on surveys or sales statistics, (c) evidence of "high" intake from bio monitoring data for the general population or for sub-populations/geographical areas, (d) compounds with high exposures relative to their reference values (i.e. ADI, ARfD), (e) CRA carried out elsewhere showing possible unacceptable exposure, (f) high number of compounds (e.g. 5 or more) in a group, and (g) predictions of upward future trends in use of pesticides.

The second Opinion (EFSA, 2009) elaborated a case study in which a method for taking account of cumulative effects was applied to a group of pesticides with similar chemical structures, the triazole fungicides. The Opinion proved to be very valuable in testing the methodology and identifying the necessary steps to be taken before the methodology could be recommended and applied on a routine basis. The PPR Panel concluded that previously proposed criteria can be simplified by starting with a CAG that is as refined as the data allows it to be and by using the same criteria in all steps of the assessment. The PPR Panel also concluded that, although a tiered approach is an appropriate way to address cumulative dietary risk assessment, it cannot yet be applied on a routine basis. The basis for, and establishment of, relevant CAGs needs to be provided first. The Panel also highlighted the difficulties in defining CAGs and in finding a consensus at the international level on the criteria to be used for establishing CAGs for active substances in pesticide formulations.

For the purpose of the present opinion, and in recognition of the mandate defined by EFSA, the PPR Panel had to clearly distinguish between the approaches used for CRA of pesticides and those required during the process of MRL setting. The Panel recognised that tiered, exposure assessment-driven approaches are powerful tools for risk assessment but are difficult to apply in the context of establishing MRLs. Since in principle (although rarely in practice) every authorised pesticide may occur in a food item, the grouping of active substances in CAGs for the purpose of MRL setting cannot be driven by exposure assessments. Instead, grouping has to be based on criteria solely derived from the intrinsic properties of the chemicals under consideration. These may include chemical structure, mode of action or the induction of common adverse effects. The Panel recognised that such grouping criteria and CAGs may also be of use in exposure assessment-driven CRA, especially at higher tiers of the analysis.

In the present opinion, the PPR Panel makes an effort to define criteria and a methodology for the grouping of active substances in CAGs for the purpose of taking account of cumulative effects during the setting of MRLs. In doing so, the Panel recognised that grouping criteria based on overly narrow definitions of modes of action or chemical structural criteria might miss substances that also contribute to a combined effect. This became evident as new data in experimental studies appeared in the literature published after the latter Opinion (EFSA, 2009). These studies showed that combinations of chemicals with shared toxicity but diverse modes of action also exhibited joint effects (reviewed in Kortenkamp et al., 2009, 2012).

In the light of this evidence, the Panel took a new approach in defining CAGs. This approach takes as its starting point the common or shared adverse effects of components in a mixture. Thus, the Panel proposed CAGs for selected shared adverse effects of active substances in pesticide formulations, also called specific effects in this opinion. As more information about modes of action of active substances becomes available, these groupings can be further refined through consideration on the possible joint effects following combination of chemicals with shared toxicity, but diverse mode of action. During the preparation of this Opinion it became obvious that the information available from pesticide Draft Assessment Reports (DARs) and the literature is in many cases not adequate to make conclusions regarding the mode of action. To deal with this difficulty in a pragmatic way when defining CAGs, two main options were identified as follows. In what might be termed an "inclusion approach", evidence is sought that a pesticide acts according to a specific mode of action before it can be included in a CAG. Alternatively, a so-called "exclusion approach" can be applied where decisions on forming CAGs are based on a weight of evidence and analysis of the nature of the toxicological effects even if a mode of action has not been clearly established. A compound is excluded from the CAG only if it can be shown that it does not exhibit the shared toxic effect (specific effect).

The Panel recognised that the "inclusion approach" could result in an underestimation of the cumulative risk while conversely the "exclusion approach" could result in an overestimation of the cumulative risk. The Panel felt that the choice of approach was essentially a risk management decision and therefore the European Commission was consulted to obtain guidance on this question.

The direction from the Commission was as follows: In the case of absence of information it is certainly not justified to assume that chemicals have no common mechanism of action, especially not when these are chemically related substances. Incidentally, chemically unrelated substances can also have a common mechanism or could show dose addition for some toxic effects even without a common mechanism. The direction given by the risk managers of the European Commission was in favour of the "exclusion approach". Consequently, this approach was adopted in the present opinion. The applicability of this approach in other areas under the EFSA remit remains to be explored.

In preparing the present opinion, EFSA commissioned a project with the aim of identifying adverse effects relevant for CRA, and of forming a basis for CAGs of pesticide active substances authorised in the EU. The results of this project were published by EFSA in January 2012 (DTU, 2012) and formed the starting point for this opinion. Subsequently, the PPR Panel established the need to further review the data collection provided by DTU. This led to the commissioning of a second project on data collection for specific organs/organ systems, and which was published in 2013 (ANSES/ICPS/RIVM, 2013).

Finally, the PPR Panel acknowledges that, according to EU residue monitoring programmes, some consumer exposure may occur from residues of non-approved pesticides. Pesticides not authorised in EU but where import tolerances exist should in the future also be included in CAG. However, in the present Opinion these substances were not evaluated for grouping due to the lack of recent and suitable peer-reviewed data.

To make the present Opinion relevant in a regulatory context, its scope was adjusted to the development of a general grouping methodology applicable to all organ and organ systems. However, this methodology was initially applied to the nervous system and the thyroid system.

Although not presented in this opinion, substantial work has been carried out to define CAGs for additional organ and organ systems and could seed continuation of the CRA in the future.

This Opinion might also inform cumulative exposures by multiple routes and to a wide variety of chemicals, not only active substances in pesticide formulations.

3. Overview on grouping approaches for chemicals

CRA often begins with the identification of chemicals that should be grouped together and subjected to joint risk assessment. Traditionally, chemicals regarded as having the ability to induce a common toxic effect by a common mechanism of toxicity have been considered together. This requirement for "similarity in mode (or mechanism) of action" is rooted in a specific interpretation of the mixture assessment concept of dose addition. However, as more and more experimental data with mixtures of a wide variety of chemicals became available, the practical applicability of criteria for similarity of action has come under scrutiny. In this section, the Panel briefly discusses notions of similar action, how they link with mixture assessment concepts, and summarises practices for the grouping of chemicals for CRA.

3.1. Similar and dissimilar joint action - the underpinnings of the mixture assessment concepts of dose addition and independent action

Dose addition, also known as concentration addition, is based on the idea that all components in a mixture behave as if they were simple dilutions of one another. Although, the original formulation of dose addition by Loewe and Muischneck (1926) contains nothing that relates dose addition to mechanisms, the dilution concept is often taken to mean that dose addition is only applicable to mixtures of compounds with a similar mechanism of action. There is good evidence (Kortenkamp et al., 2009) that combinations of chemicals which interact with the same well-defined molecular target indeed follow the dilution principles of dose addition: each component can be replaced totally or in part by an equal fraction of an equi-effective concentration (e.g. an EC_{50}) of another, without changing the overall combined effect.

The alternative mixture assessment concept of independent action, sometimes also termed response addition, effect multiplication or Abbotts Rule, conceptualises mixture effects in a different way. It assumes that a combination effect can be calculated from the responses of the individual mixture components by following the statistical concept of independent random events (Bliss, 1939). Although independent action is also applicable to chemicals that act through similar modes of action when these are administered sequentially, the principles of independence of action are thought to be met by substances with strictly dissimilar modes of action only when exposures occur simultaneously.

These distinctions are relevant when it comes to deciding which of the two concepts should be used for the assessment of a specific mixture. In the past, independent action was often held to be applicable when the similarity criteria of dose addition appeared to be violated (COT, 2002). By implicitly taking "dissimilar action" as the simple negation of "similar action", it was then assumed that independent action must apply, even without further proof that the underlying mechanisms satisfy the dissimilarity criterion.

What complicates decisions about the application of dose addition or independent action is a lack of reliable criteria for similarity of mechanisms and modes of action. Accordingly, opinions about what should qualify for "similarity" differ considerably. While it is widely accepted to regard mixtures composed of chemicals that act on the same molecular structure as acting similarly in accordance with dose addition, the issue is complicated by observations that dose addition sometimes also provides good descriptions of experimentally observed combination effects when strict mechanism-based criteria of similarity are not met (Kortenkamp, et al., 2009). This has lent support to the idea that notions of similarity of action are also applicable when all mixture components produce the same phenomenological effect. On the other hand, that approach could turn out to be inappropriate for some combinations of chemicals when they induce a common effect through distinct molecular mechanisms. Conversely, demands for a strict mechanistic similarity criterion can mean that very few chemicals actually qualify for the inclusion in a CAG.

This would leave many other chemicals, which also produce the same adverse outcome unaccounted for, with the consequence of underestimating combined risks. Others hold the middle view that interactions with the same site, tissue, or target organ should qualify for similarity (US EPA 1986, 1989; Mileson et al., 1998).

As a way of dealing with these difficulties, IPCS (2009) proposed to use dose addition as the default concept in cumulative risk assessment until evidence to the contrary, in support of dissimilar modes of action, becomes available.

3.2. Similarity of action as a grouping criterion

The problem regarding the choice of dose addition or independent action for the assessment of specific mixtures can be solved pragmatically by evaluating experimental data using both concepts side-by-side, with the aim of investigating which concept produces the best approximations of the observed effects. Such comparative evaluations have revealed a great deal about the applicability of dose addition and independent action (Kortenkamp et al., 2009, 2012).

However, *post hoc* analyses of this kind are not an option when it comes to judging whether substances whose combined effects are untested will produce a combination effect, let alone whether this combination effect can be approximated by dose addition and is in line with notions of similarity of joint action. In principle, such judgements, and the corresponding grouping decisions, can be made by considering the effect profile of each substance individually in terms of common adverse outcomes.

The alternative option can be to use narrower criteria of similarity, based on common mechanisms and molecular targets. In practice, this has often meant that chemicals with shared structural characteristics were grouped together. The Panel recognises that any grouping effort requires information about mechanisms or modes of action in relation to multiple toxic endpoints. Unfortunately, such information is often not available for many pesticides. The data requirements for the approval of pesticides, as laid down in Commission Regulation (EU) No 283/2013[8], are not geared towards meeting the requirements of CRA.

To gain an impression of how other agencies and institutions deal with the issue of grouping pesticides and other chemicals for the purpose of CRA the Panel briefly reviewed examples of current practice.

3.3. Grouping approaches by other international bodies

The US Environmental Protection Agency (US EPA) has evolved one of the most elaborate regulatory frameworks for CRA. For pesticides, this derives from a mandate laid down in a clause of the US Food Quality Protection Act (FQPA). This clause requires US EPA to conduct CRA for human health effects that result from exposure to multiple chemicals with a common mechanism of toxicity.

US EPA currently conducts CRA for five groups of pesticides: organophosphorus compounds, N-methyl carbamates, s-triazines, chloroacetanilides and pyrethrins/pyrethroids.

In all these cases, CRA begins with the identification of a common mechanism group where pesticides that induce a common toxic effect by a common mechanism of toxicity are grouped together. US EPA determines that a common mechanism of toxicity exists if chemicals act in the same way in the body, i.e. the same toxic effect occurs in the same organ or tissue by essentially the same sequence of biochemical events. If necessary, temporal aspects are also considered to determine exposure durations relevant for the induction of a common toxic effect.

Common mechanism groups are then used to define common assessment groups, essentially by excluding substances whose uses, routes and pathways of exposure are deemed to contribute little to a cumulative risk.

The earliest CRA was conducted for organophosphates in 1999, with the establishment of a common mechanism group based on the ability of these pesticides to inhibit acetylcholinesterase (AChE) by phosphorylation. The CRA for organophosphates was most recently updated in 2006 (US EPA, 2006a).

[8] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. Official Journal L 93, 1-84. 3 April 2013.

A second common assessment group encompasses N-methyl carbamates which also inhibit AChE, but by carbamylation of a serine in the active centre of the enzyme (US EPA, 2007). In contrast to organophosphates, there is fairly rapid recovery of the enzyme after maximal inhibition. Presumably, this was the justification for not grouping N-methyl carbamates and organophosphates together in one assessment group.

Another assessment group is made up of s-triazine pesticides, consisting of atrazine, simazine and their common metabolites. These substances are judged to produce neuroendocrine and endocrine-related developmental toxicity by a common mechanism involving disruption of the hypothalamic-pituitary-gonadal axis (US EPA, 2006b).

Chloroacetanilide pesticides that produce tumours of the rat nasal olfactory epithelium via the cytotoxic action of common tissue metabolites are also subjected to CRA (US EPA, 2006c). The corresponding common mechanism groups consist of acetochlor, alachlor and butachlor, but only acetochlor and alachlor are subjected to CRA in a common assessment group, because butachlor has no registered uses in the USA.

The most recent cumulative assessment deals with pyrethroids and pyrethrins (US EPA, 2011). These insecticides were included in a common mechanism group based on shared structural characteristics, a common ability to interact with voltage-gated sodium channels, resulting in the disruption of membrane excitability in the nervous system, and finally neurotoxic effects characterised by two different toxicity syndromes. US EPA considered it appropriate to include *ad interim* all relevant substances together in a single CAG, selecting deltamethrin as Index Compound (IC). As low-hazard pyrethroids that do not induce the typical neurobehavioral effects at the limit dose of 5000 mg/kg bw, tetramethrin and sumithrin were not included in the common assessment group. Pyrethroids that are not detected as residues in crops were also excluded.

In settings where non-pesticide chemicals are considered (e.g. for the assessment of superfund sites; United States federal law designed to clean up sites contaminated with hazardous substances), the US (1989) has developed approaches involving multi-step procedures for the classification of chemicals into groups suitable for CRA. Here, the process begins by grouping chemicals of concern according to their potential of occurring together in the same medium and at the same time. The groupings derived in this way are then divided into subgroups defined according to their propensity to cause common toxicity by common modes of action or in terms of their capability of affecting the same target organ.

This approach has similarities to the IPCS tiered framework for CRA which uses dose addition as the default risk assessment method (IPCS, 2009; Meek et al., 2011). The IPCS framework is essentially exposure-driven. For each chemical included in the assessment, exposure and hazard assessments are conducted in a step-wise (tiered) manner, but separately. At lower tiers, fairly crude estimates can be used, and the analysis is discontinued when guidance values are not exceeded. Only when lower tier assessments signal potential risks, is the analysis refined by introducing higher quality exposure and hazard data. At lower tiers, all chemicals that co-occur in the setting under consideration can be included, irrespective of any assumptions about their modes of action. Groupings based on modes of action or mechanisms can be introduced at higher tiers, if necessary. The IPCS framework was applied in two cases studies, on polybrominated diphenyl ethers (PBDE) and surface water contaminants. Some guidance was given on factors that could be taken into account in CAG, e.g. chemical structure, identification of common potential toxicophores (structural alerts), similarity of target tissue and/or manifestations of toxicity.

A report on CRA for phthalates and other antiandrogenic chemicals, and their impacts on developmental toxicity, by the US National Research Council highlighted the shortcomings of using too narrowly focused mechanistic criteria as the basis for groupings (US NRC, 2008). The US NRC report noted that several phthalates produced joint effects with other, structurally diverse anti-androgenic substances. The NRC Panel recognised that disruption of male sexual differentiation depends on proper androgen action in foetal life and concluded that the available experimental

evidence showed that dose addition applied, despite the fact that androgen action can be diminished by a variety of mechanisms. Independent action-based prediction consistently led to underestimations of combination effects of these chemicals. On the basis of these observations, the US NRC proposed that a physiological concept based on common adverse outcomes should underpin decisions about the similarity of action of mixture components. It recognised that such similarity criteria go far beyond criteria derived from similarities in chemical structures.

3.4. Activities in the EU

The non-food Committees of DG SANCO (Scientific Committee on Consumer Safety-SCCS; Scientific Committee on Health and Environmental Risks-SCHER; Scientific Committee on Emerging and Newly Identified Health Risks-SCENIHR) dealt with issues relevant to the grouping of chemicals in CRA in 2009 with an Opinion on the assessment of the antibiotic resistance effects of biocides (SCENIHR, 2009). One problem within the Biocides Directive 98/8/EC[9] is that cumulative risks that arise from the use of an active substance outside the scope of the Directive (e.g. in plant protection products, consumer products, human or veterinary medicines, food hygiene, etc.) are not addressed in the evaluation process. This was regarded as particularly problematic for the selection of antimicrobial resistant bacterial strains, which can arise from the combined action of different substances with diverse mechanisms when these substances are in different regulatory domains. However, with the new EU Biocides Regulation EC (No) 528/2012[10] further provisions with regard to cumulative assessments of biocides have been introduced taking into account such effects for the authorisation process. Also a guidance document addressing the issue of cumulative effects is currently being developed.

At the request of the European Commission, the non-food Committees of DG SANCO (SCCS, SCHER, SCENIHR) produced a joint Opinion on the Toxicity and Assessment of Chemical Mixtures in 2011. Their conclusions broadly agreed with the EFSA PPR Opinion (2008). Relevant to the topic under consideration here is the observation of the committees that there are a limited number of chemicals for which sufficient information on their mode of action is available. An agreed inventory of mode of actions, as well as a defined set of criteria for ways of characterising or predicting modes of action in data-poor situations is missing. The committees also suggested the use of dose addition as a default in cases where evidence to the contrary is not available.

3.5. The grouping approach adopted by the PPR Panel - general considerations

In defining CAGs to take cumulative effects into account in the decision on applications concerning MRLs, the Panel has chosen to follow an approach that focuses on shared toxicity profiles of pesticides. In view of the mandate and the regulatory context in the EU, a purely exposure-driven grouping procedure was considered inappropriate, mainly because all authorised active substances used in pesticide formulations can theoretically occur in human diet.

However, when the objective is to conduct risk assessments for combinations of pesticide residues in food, an exposure-driven approach according to the tiering principles developed by IPCS (2009) and also in the previous opinions of the PPR Panel (EFSA 2008, 2009) would be the method of choice.

The Panel recognises that the use of the quite narrow mechanistic grouping criteria by US EPA is mandated by the US Food Quality Protection Act[11] with its focus on subjecting pesticides with a common mechanism of action to CRA. Such restrictions do not exist in the EU. The Panel shares concerns that quite narrowly defined mechanistic grouping criteria might leave out pesticides that contribute to common toxic effects. In the interest of consumer protection, a grouping approach which

[9] Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. Official Journal L 123 , 24/04/1998 P. 1 – 63.
[10] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal L 167, 27.6. 2012. 1- 123.
[11] Available from: http://www.epa.gov/pesticides/regulating/laws/fqpa/gpogate.pdf

emphasises common adverse outcomes was adopted for the purposes of MRL setting. Where the relevant data were available, more refined groupings based on modes of action were made.

In many cases, however, the lack of relevant data about modes of action and pathways leading to common adverse outcomes made it necessary to use common target organs as proxy. In choosing this approach, the Panel does not exclude that common effects in tissues, organs or physiological systems could be the result of different toxicity pathways. A second assumption underpinning this grouping approach was that pesticides with common toxicity pathways will elicit common toxic effects in an additive fashion.

Accordingly, the groupings were based on common toxic effects regardless of whether these represented the critical effects that drive the risk assessment of single pesticides, i.e. the effects from which health-based guidance values such as acceptable daily intake (ADI) or acute reference dose (ARfD) are derived.

4. Summary of the previous opinions - lessons learnt

In 2008, the PPR Panel adopted an Opinion to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risks from pesticides to human health with a view to set MRLs for those pesticides in the frame of Regulation (EC) No 396/2005.

Having considered the evidence on the different forms of combined toxicity and their potential relevance to risk assessment for pesticide residues at the levels occurring in food, the PPR Panel limited this Opinion to the possible impact of dose-addition. In particular, the PPR Panel noted that although toxic interactions from pesticide residues in food cannot be ruled out, there is no empirical evidence for their occurrence at the expected levels of exposure from pesticide residues in food. This approach has since then been supported by the analysis carried out for the Scientific Report on the investigation of the state of the art of science on combined actions in food through dissimilar modes of action and proposal for science-based approach for performing related CRA (Kortenkamp et al., 2012).

The key steps leading to toxic effects and criteria by which to define a cumulative assessment group as proposed by the US EPA were outlined in the Opinion of 2008. These steps included the following;

1. Preliminary identification of a candidate set of substances that might cause a common toxic effect by a common mode of action. This preliminary grouping is based on one or more of the following criteria:
   a. chemical structure. This can be explored by substructure searches in databases for toxophore (or a metabolic precursor of a toxophore), core molecular structure, functional groups;
   b. mechanism of pesticidal action. This is considered informative because it is not uncommon that pesticides are toxic to humans through a mechanism that is similar to that of their activity against their target pests;
   c. general mode/mechanism of mammalian toxicity;
   d. a particular toxic effect. It is conceivable that similar toxic effects by different compounds might be caused via a common mode/mechanism. This criterion might allow the identification of structurally unrelated substances that act by the same mode of action. It is emphasized that non-specific effects such as body weight changes or death can result from many unrelated factors and consequently are of limited value in identifying potential candidate substances for a common mode/mechanism group.

2. Identify those substances from step 1 that cause a common toxic effect(s). This step allows a first refinement of the preliminary grouping described above. This is to be performed by detailed evaluation of available toxicology data for each substance and those not causing a common (i.e. concordant in both site and nature) toxic effect are excluded.

3. Determine the toxic mode/mechanism of action by which each substance causes a common toxic effect.

4. Compare the mechanisms of toxicity/modes of action of the different substances.

5. Refine groupings by excluding substances that cause a common toxic effect by a different mechanism/mode of action.

The PPR Panel concludes that full consideration of all of these criteria will provide the most sound and robust grouping. However, such a detailed evaluation up to the last step might not be necessary or even possible in all cases. For the purposes of risk assessment, compounds might be grouped even in the absence of such detailed data and thus on the basis of a less refined evaluation of the mode of action (e.g. based only on target organ toxicity).

An additional consideration arises from evidence in the literature that certain reproductive toxicants show dose-addition even if they do not share the same primary molecular target (Kortenkamp, 2007 and papers there reviewed). Therefore, the issue is the definition of the concept of common mode of action and what this would mean for these reproductive toxicants. For instance, compounds affecting male sexual development via interference with steroid synthesis and not by antagonism of the androgen receptor would not be grouped according to a narrow definition of mode of action whereas it has been shown that a mixture of such compounds results in a dose-additive effect (Gray et al., 2001; Hotchkiss et al., 2004; Jacobsen et al., 2012; Hass et al., 2012). Similar considerations can be applied to estrogenic or estrogen-like chemicals (Picard, 2003). Therefore, it appears that in these cases the criterion for grouping should rather be that of a common phenomenological effect (e.g. altered ano-genital distance for antiandrogens) (Kortenkamp, 2007).

Following this opinion, an exercise to test the proposed methodology on CRA was applied to a group of triazole fungicides, and the results were reported in a separate Opinion with any suggested refinements necessary to the methodology. This Opinion of the PPR Panel was adopted in June 2009. The triazole exercise was not to be considered as the definitive EU risk assessment of the combined triazole group, but rather as a worked example, which illustrated and tested the proposed methodology.

Thus, in the triazole Opinion the proposed CRA methodology was applied to a selected group of substances. The grouping was based on the structure of the substances, i.e. the triazole ring, the pesticidal mode of action i.e. the inhibition of the sterol biosynthesis (C14-demethylase), and the mode of action that cause a common toxic effect collected from DARs, JMPR reports or US EPA assessments. For acute assessment, cranio-facial malformations induced by triazoles were ascribed to a common mechanism of toxicity. In the chronic assessment the triazoles liver toxicity was the common adverse outcome used. A common mode of action has not been established for these effects and further refinement of the grouping was not possible for this group of pesticides.

The previous opinions (EFSA, 2008, 2009) dealt with possible models for a CRA, encompassing hazard and exposure assessment and proposing a tiered approach. According to the Terms of Reference, the current Opinion deals therefore only with hazard assessment and the identification of CAGs based on toxicological profiles of pesticides.

5. Supporting projects

5.1. Identification of cumulative assessment groups for pesticides

EFSA commissioned the Danish Technical University (DTU) to form Common Assessment Groups based on organ specific effects for active substances included in Annex I of Directive 91/414/EEC[12] (up to 31st of May 2009). Active substances were considered for CRA if these were chemically well-defined and if there was adequate toxicological data available for the substances of interest. Active substances not considered for grouping were micro-organisms, complex and poorly defined mixtures of chemicals (e.g. pheromones), those with insufficient toxicological data, and substances considered as having no harmful effects on human and animal health (i.e. with no established reference value). As a result, 224 of the 344 active substances in Annex I were considered relevant by DTU.

Information on toxicological effects and modes/mechanisms of action was collected from EU peer reviewed documents (e.g. draft assessment reports (DARs), ECCO and EPCO assessments). Additional relevant toxicological information was collected from Joint FAO/WHO Meeting on Pesticide Residues (JMPR) reports and from the open literature.

The criteria for grouping proposed by the PPR Panel in 2008 was the starting point for the outsourced work performed at DTU. It was soon recognised during the review of the active substances for inclusion in cumulative assessment groups, that there was often little or no data available on mode of action but that many substances affected the same target organ and/or cell population. Thus, the grouping methodology proposed by DTU was based on phenomenological effects of the substances and the following approach was applied:

- CAG level 1: Toxicological target organ
- CAG level 2: Common specific phenomenological effect
- CAG level 3: Common mode of action
- CAG level 4: Common mechanism of action

CAG level 1. The initial screening of toxic effect(s) on target organ/organ systems resulted in an allocation of active substances into CAGs at level 1. Many of the substances showed effects on several organ/organ systems and were therefore allocated into more than one CAG.

The following organ systems were suggested for CRA: adrenal gland, bone marrow, bones/skeleton, cardiovascular system, eye, gallbladder, haematological system, kidney, liver, muscles, nervous system, parathyroid gland, reproductive system and developmental toxicity, spleen, thyroid and urinary bladder. CAGs for CRA were not recommended for the gastrointestinal tract, immune system, lung, lymph node, pancreas, pituitary gland, salivary gland, skin and thymus.

CAG level 2. This group was proposed to include substances that exert a specific phenomenological effect on the target organ/organ system in question without any consideration of mode of action. In many cases, an active substance was found to lead to several specific phenomenological effects in a given target organ/organ system. The reason for this is that the proposed different specific effects could be considered as representing a continuum of pathological findings for a given target organ/organ system.

Wherever possible, NOAELs and LOAELs were noted in each study for each effect. For many of the specific effects, NOAELs and LOAELs were identical to or even higher than the NOAEL and LOAEL of the study used to set regulatory reference values.

[12] Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. Official Journal L 230, 1-290. 19 August 1991.

In assessing adversity, some effects are considered in single substance assessment as being non-adverse if they occur below a certain limit. For CRA, the DTU considered it not relevant to distinguish between adverse and non-adverse effects based on limits because the accumulated increase caused by several substances, each contributing with an increase under the specified limit, may exceed the limits.

There were cases in which effects for individual substances were observed after a short period of exposure, but became undetectable in the course of prolonged exposure. Such effects might be considered adaptive for single substance assessment, but DTU considered that it could not be excluded that an organism will not be able to adapt if exposure occurs to several substances simultaneously. Accordingly, an effect was regarded as being adverse even if it was no longer measurable after prolonged exposure.

CAG level 3. This group was proposed when information or a hypothesis was available on a common mode of action behind a specific effect. For a number of specific effects data did not allow for further allocation of substances into CAGs at level 3. For some of the organ systems, for example for parts of the endocrine system and the nervous system, modes of action were proposed.

CAG level 4. For some of the active substances, mainly *in vitro* data reported in the open literature could support a further refinement of the mode of action at level 3, and hence a specific mechanism of action could be proposed. For example, active substances that are acetylcholinesterase inhibitors (and thus modulate the cholinergic system; CAG level 3) or active substances being antagonistic to the androgen receptor (and thus affecting for example the male reproductive organs via anti-androgenic mode of action; CAG level 3) were allocated to a CAG at level 4.

All together, this resulted in (*i*) a number of groups that could not be further refined into CAGs at level 3 and 4 because data on mode/mechanism of action for many endpoints were inconclusive, (*ii*) the groups proposed were, therefore, mostly for phenomenological effects, i.e. CAG level 2, (*iii*) due to lack of mechanistic data many of the proposed groups contain several substances, and (*iv*) many of the substances caused several effects and were therefore allocated into more than one CAG of the same level. Since many active substances appear in many groups, the data entries for performing CRA are of considerable magnitude.

In association with an external scientific report, published in April 2012 by the DTU, a database (CAPEG) was developed. The database fields contain toxic effects and respective indicators that can be used for establishing common assessment groups on which a CRA could be performed. Also, NOAELs and LOAELs were listed for each effect in the database.

The DTU collection and assessment of the active substances included in Annex I prior to 31st of May 2009 was evaluated by the present working group, which concluded that further consolidation of the outcome of the DTU evaluation was needed. This consolidation was performed jointly by working group members and staff of the Pesticides Unit at EFSA.

5.2. Toxicological data analysis for effects on the liver, on the nervous system and on reproduction and development

As a result of the consolidation exercise performed on the DTU study output, EFSA launched a call for tender "Toxicological data analysis to support grouping of pesticide active substances for CRA of effects on the liver, on the nervous system and on reproduction and development" (ANSES/ICPS/RIVM, 2013), which was awarded to a consortium of the International Centre for Pesticides and Health Risk Prevention (ICPS; Italy), the National Institute for Public Health and the Environment (RIVM; The Netherlands), and the French Agency for Food, Environmental and Occupational Health and Safety (ANSES; France) which lead the project. In addition to the pesticides evaluated by DTU, 60 new active substances were added to Annex I from 31st of May 2009 until 1st of January 2012, and 3 pesticides (flurtamone, oxadiargyl and pyridate) not screened by the DTU were also evaluated.

In total, 260 substances were found to have reproductive and developmental effects by ANSES, 68 substances were found to be neurotoxic by RIVM, and 244 substances were found to cause effects on the liver and biliary system, including the gallbladder by ICPS. All the findings (endpoints) that were indicated in the contract as indicative for those effects have been reported for each substance, with their respective NOAELs/LOAELs. The selection of NOAELs and LOAELs was performed, as requested by EFSA, without any interpretation of whether an effect is to be considered adverse or not adverse. In the report, established or postulated mode of action was reported as well as reference to possible sources of information in this respect, which mostly included the open literature. No in-depth analysis of proposed or postulated mode of action was performed. The authors of the report recommended that further work on the establishment of cumulative assessment groups for these organ systems should be done by specialists in these areas.

A comprehensive database with the information collected was provided by the contractors. The data collection of neurotoxic effects was the basis for the proposal of cumulative assessment groups for the nervous system in this opinion.

6. Grouping methodology

This section describes the criteria developed for selecting specific effects of pesticidal active substances as a basis to determine cumulative assessment groups. The methodology has been developed on the basis of toxicity studies involving oral exposure to take into account cumulative effects on human health potentially resulting from exposure to a combination of active substances as described in the Regulation (EC) 396/2005 on maximum levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. The methodology developed might be applicable also for dietary and non-dietary CRA. This general methodology deals with grouping of active substances showing common specific effects and was initially applied for grouping of active substances affecting the nervous system and the thyroid system. In the future, the approach should be implemented upon dealing with specific issues with regard to all other relevant organs and organ systems.

During the process of the grouping, the Working Group took note of any demonstrated modes/mechanisms of action and also on how some effects may be related to each other. Wherever possible, the identified effects were qualified with regard to their nature as either acute or chronic for the organ system evaluated.

The PPR Panel notes that the CAGs in this Opinion based on the outlined approach would be provisional. Groups are expected to change if new pesticides are included, if new data allows exclusion of pesticides from a group, or if new mechanistic data provide more evidence for a specific mode of action as a more appropriate basis for a CAG.

In current practice, mechanistic information, which allows understanding of the ultimate biochemical events (or mode of action) causing the different toxic effects, is normally not required in regulatory toxicology of pesticides and is therefore generally lacking.

Consequently, in this Opinion an approach was followed whereby active substances were grouped based on the occurrence of toxicologically relevant and unambiguously defined effects on the target organ i.e. on specific effects, even if the underlying initial biochemical events causing these effects have not (yet) been demonstrated experimentally.

This methodology elaborates CAGs at several levels; CAG level 1 at organ/organ system level; CAG level 2 based on specific phenomenological effects; and potentially further refinement based on information about the specific mode of action.

This approach follows the recommendations given by the Commission to the PPR Panel with regard to grouping, suggesting that in the absence of information a precautionary approach should be followed i.e. an "exclusion approach". This may result in the grouping of substances in the same CAG, although they might exert their effects through dissimilar mode of actions.

The methodology developed comprises four main steps:

6.1. Step 1: Identification of specific effects appropriate for grouping for CRA

This section describes how the effects observed in toxicological studies should be selected as relevant effects for CRA.

The identification of specific effects result from a screening procedure as described below.

6.1.1. Step 1.1: Exclusion of local effects

Local effects mainly refer to health effects that take place at the point or area of contact by a chemical-physical action rather than a biochemical action. They can be induced for instance by caustic substances or by corrosive materials, e.g. internally in the gastrointestinal or externally on skin and in eyes.

Local effects are not produced by the potentially absorbed dose. Thus, they are not systemic effects and do not form the basis for the setting of reference values for dietary risk assessment. Local effects are, therefore, not to be considered as specific effects of combined toxicity to support MRL setting.

6.1.2. Step 1.2: Exclusion of non-adverse effects

The distinction between adverse and non-adverse observed effects is crucial to ensure proper hazard identification and is a matter of expert judgement.

In discriminating between an adverse and a non-adverse effect, consideration is given to its adaptive nature, its transient or persistent nature, its magnitude, its association with other alterations, whether it is a precursor to a more relevant effect, and its impact on the overall function of the organism (Lewis et al., 2002).

Adaptive responses may not be considered adverse for single exposure. They consist of the initial response of the organism to maintain homeostasis, and can be defined as those biological effects that do not cause biochemical, physiological, and morphological changes that affect the general well-being, growth development or lifespan of the organism (Lewis et al., 2002). In general, effects that are non-adverse do not qualify for the basis of setting either a NOAEL or a LOAEL (JMPR, 2005).

As recommended in the DTU report, some effects which are considered in single substance assessment as being non-adverse if occurring below a certain limit of magnitude were considered in the proposed methodology as being potentially adverse and thus relevant within the context of CRA. There are cases in which effects for individual substances are observed after a short period of exposure, but become undetectable in the course of prolonged exposure. Such effects might be considered adaptive for single substance assessment. However, it cannot be excluded that an organism's capacity for adaptation will be exhausted if there is exposure to several substances simultaneously. Accordingly, an effect was regarded as being adverse even if it becomes undetectable after prolonged exposure.

6.1.3. Step 1.3: Exclusion of effects not relevant for human risk assessment

Effects observed in animal studies have to be assessed also for their relevance to humans before being selected as a basis for CRA. Effects not considered as relevant for human risk assessment should not be used for inclusion of chemicals in CAGs. In the absence of specific data demonstrating non-relevance, the default assumption is that effects are relevant to humans.

The expression of toxicity in mammalian systems is dependent on a sequence of key events taking place. Thus, for a particular effect, it is necessary to justify the *irrelevance* to humans. This might be possible for effects observed in organs of laboratory animals where there is no equivalent in humans and should therefore not be considered for CRA (e.g. the Harderian and Zymbal glands of rats where tumours can occur).

6.1.4. Step 1.4: Evaluation of the unambiguous nature of the effect

When performing CRA, it is important that a specific effect considered as relevant is unambiguous and well-defined in terms of site and nature. In some instances it is known that the chain of events leading to an effect is caused by a single biochemical event (e.g. acetylcholinesterase inhibition). When an adverse effect appears in isolation and not in relation to other effects/parameters or patterns this should be interpreted very carefully.

Similarly also after long term exposure any results must be interpreted carefully since they can be confounded with effects caused by aging or other physiological processes.

6.1.5. Step 1.5: Identification of non-specific effects

Non-specific effects resulting from severe toxicity are not considered relevant for CRA in accordance to the DTU report, e.g. acute clinical effects in the presence of mortality.

However, non-specific effects, being the consequence of a primary effect, in certain cases can be used for CRA, if such non-specific effects are not the result of severe toxicity and if a mode of action is identified, showing that the non-specific effect, together with the specific effects, might contribute to a common outcome. Therefore non-specific effects can also lead to inclusion of substances in groups with possibly different modes of action, leading to conservative grouping.

As an example, a reduction in circulating thyroid hormone levels may be due to different modes of action, some of which directly affect the thyroid itself (e.g. via inhibition of thyroid hormone synthesis). However, a decrease in circulating thyroid hormone levels may also occur as a consequence of enhanced hormone metabolism, owing to enzyme induction mainly in the liver. In this case, a challenge to the thyroid hormone system would be regarded as "non-specific" being "indirect" or a "secondary"consequence of the specific effect of enzyme induction. However, if several modes of action (direct and indirect) feed into a common adverse outcome (e.g. decrease in circulating levels of hormones), it appears feasible not to disregard the indirect mode of action for CRA. This is exemplified in further detail in the Appendix E on grouping for substances affecting the thyroid system. It is noted that only in rare cases mechanistic information could be derived from the DARs.

Overall, non-specific effects in terms of mode of action need to be assessed on a case by case basis following a weight of evidence approach and expert judgement.

6.2. Step 2: Characterisation of the specific effects

After identification of the specific effects of relevance for cumulative assessment grouping for a particular organ/system, it is necessary to characterise these effects, in a consistent way, to decide which sub-groups might be distinguished on a phenomenological basis. In particular, it should be investigated whether or not several effects may be interrelated. Several different effects may have a common general mechanistic basis, or may be the result of toxicity towards a common general target structure. For example, toxicity to the autonomic nervous system, identified in this Opinion as a specific effect, may manifest itself in form of a number of different observations, designated in this Opinion as indicators, including miosis, salivation, lacrimation and urination. Furthermore, some indicators for a specific effect may be linked to one another by a chain of events. As an example, a decrease in circulating thyroid hormone levels would be expected to lead to an increase in TSH levels, which would result in stimulation of thyroid follicular cells. Prolonged stimulation of follicular cells in the rat may lead to development of follicular cell tumours. If several such observations (or indicators) are connected to one another by a chain of events, it is recommended to establish the order of their sequential occurrence (as a list of indicators for the CAG).

In cases in which different indicators are interrelated, these effects may be used to define a common phenomenological sub-group. If data on underlying modes of action leading to specific effects are available, they should be considered to decide whether a further refinement of grouping may be feasible. It is noted, however, that information on the causal mode/mechanism of action is missing for the majority of the effects observed in toxicological studies.

The characterisation of the specific effect, the underlying mode of action leading to it, and the possible interrelationship between different specific effects is done case by case and is a matter of expert judgement.

6.3. Step 3: Data Collection

After identification and characterization of specific effects and their respective indicators (measured endpoints) for each target organ/organ system, the available toxicological data for each active substance should be collected. Active substances not considered were micro-organisms, complex and poorly defined mixtures of chemicals (e.g. pheromones), those with insufficient toxicological data, and substances considered as having no harmful effects on human and animal health (i.e. with no established reference value). As a result, 224 of the 344 active substances in Annex I (up to 31st of May 2009) were selected by DTU. The same criteria were applied to the active substances included in Annex I from 31st of May 2009 until 1st of January 2012, resulting in 60 new substances to be screened for CRA. Three substances previously excluded by DTU were also added to the list of active substances to be screened. The active substances screened for neurotoxicity and thyroid toxicity are presented in Appendix H.

Based on a screening of relevant regulatory toxicity studies, i.e. those studies considered acceptable during the peer review of active substances, the retrieved data should include all the relevant indicators of a specific effect observed in one or more studies. Specifically, the following information should be collected for grouping and further CRA.

- Name of the active substance
- Target organ/organ system
- Chemical class
- Chemical name (IUPAC)
- CAS number
- Pesticidal mode of action
- Study type
- Species
- Strain
- Route of administration
- Type of administration
- Indicator (measured endpoint) of a possible common specific effect
- Specific NO(A)EL acute (mg/kg bw): after single administration
- Specific LO(A)EL acute (mg/kg bw): after single administration
- Remarks related to the indicator (used to insert further details about the effect)
- Remarks related to the study (acceptability, GLP, Guidelines, overall NOAEL, etc.)
- Specific NO(A)EL rpd (mg/kg bw/day): after repeated administration
- Specific LO(A)EL rpd (mg/kg bw/day): after repeated administration
- Remarks related to the indicator (used to insert further details about the effect)
- Remarks related to the study (acceptability, GLP, Guidelines, overall NOAEL, etc.)
- Mode/mechanism of action (MoA); known/unknown/presumed
- Remarks on mode/mechanism of action
- Reference to the study (author, year)
- Source of information to the toxicity studies
- Year of evaluation (publication of EFSA conclusion/Commission's review report)
- Remarks on EFSA conclusion (derivation of the ADI and ARfD, etc)

For dietary CRA, only toxicity studies performed by oral administration (diet, capsule, gavage) should be considered.

In cases in which different specific effects are interrelated, it is recommended to establish the most appropriate indicator of a specific toxicological response in the most appropriate/sensitive species. After ranking all related specific effects serving as indicators of a specific toxicological response in sequential order, it may be that data for all indicators are not available for all the pesticides causing

this specific response. In particular, data to establish the NOAEL for the specific effect may be lacking for the most sensitive indicator. In this case, the NOAEL should be established on the basis of the alteration of the second-most sensitive indicator.

6.4. Step 4: Cumulative Assessment Groups

Establishment of CAGs for effects on the nervous system was chosen since these effects were a priori considered as highly relevant with regard to potential cumulative effects and considering the fact that the data collection could discriminate CAGs into acute and chronic effects. Effects on the thyroid system were, on the other hand, considered since the thyroid is a frequent target of pesticide toxicity and the effects may be an example of potential toxicity to the endocrine system.

The PPR Panel has already carried out substantial work on the establishment of many CAGs in addition to those already presented in the current opinion. Because elaborations on these additional CAGs have not been completed they are not mentioned in the opinion. Nevertheless, this work should be considered as a starting point for further work on CAGs.

7. Grouping methodology for the nervous system

7.1. Hazard identification and characterisation for neurotoxicity (Step 1 and 2)

In the DTU report (2012), the first level for CAG was defined as 'Toxicity to the nervous system' as the nervous system was identified as a relevant target for some active substances.

The methodology for hazard identification and characterisation of toxic effects of active substances on the nervous system combines information provided by DTU (DTU, 2012) and RIVM (ANSES/ICPS/RIVM, 2013). These were further confronted with information from US EPA and IPCS/WHO reports. The three major functional divisions of the nervous system (motor, sensory and autonomic) were identified as potential targets for these substances along with the neurochemistry and neuropathology parameters.

CAG level 1 (organ/organ systems level)

The most conservative level of grouping on a physiological basis comprised all substances affecting the nervous system either central or peripheral.

CAG level 2 (refinement at the effect level)

Further refinement of grouping on the phenomenological level was based on different general targets concerning neurotoxicity and was therefore recommended for neurotoxic hazard characterisation:

- Effects on motor division (e.g. locomotor activity, muscle strength, coordination and equilibrium).
- Effects on sensory division (e.g. including reflex action or sensory-motor responses and neurophysiological assays).
- Effects on autonomic division (e.g. cholinergic modulation).
- Neurochemical effects (e.g. brain or erythrocyte acetylcholinesterase inhibition).
- Neuropathological effects (mainly axonal and myelin degeneration).

The most appropriate indicators for the specific effects are shown in the table for hazard identification and characterisation (Table 1).

Developmental neurotoxicity (DNT) should also be considered for hazard characterization. However, specific DNT testing has not been conducted according to OECD guideline 426 since it was developed in 2007 (OECD, 2007). Accordingly, the CAGs for DNT effects cannot be considered conclusive because the experimental studies available for risk assessment were restricted to developmental and reprotoxicity testing. Since results for DNT testing were only available for a few active substances (dimethoate, fenamiphos, fipronil, malathion and molinate) DNT effects were not grouped. Also, because available toxicological data from DARs did not contain sufficient information on effects on the cognitive domain (learning and memory), this domain was not considered for grouping.

Table 1: Hazard identification/characterisation for neurotoxicity

	Hazard Identification (Step 1)							**Hazard characterisation (Step 2)**
Specific effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/ conclusions*	**Most appropriate indicator for effect** *(see Notes 1 and 3)*
Motor division	No	Yes	Yes	Yes *See also column 'Relevant for CRA'.*	No *In the absence of information suggesting such effects being related to specific effects which might be found when doing step 3).*	Unknown for pesticide active substances *Overall no general MoAs for these effects (for OPs and N-methylcarbamates modulation of cholinergic transmission – see Note 2).*	Yes *However, if these effects result only at the high dose and together with other overt signs of toxicity, there is less persuasive evidence of a direct neurotoxic effect.*	Reduced motor activity: hypoactivity, recumbency, lateral posture, etc. Increased motor activity: tremor, choreo-athetosis, hyperactivity , convulsions, etc. Muscle strength: reduced grip strength, increased or decreased muscle tone, muscle fasciculation, weakness, ptosis, inability to stand, paresis, paralysis, etc. Coordination: ataxia, abnormal gait, landing foot splay, etc.
Sensory division (including sensorimotor reactivity)	No *Except paraesthesia, see Note 4.*	Yes	Yes	Yes *See also column 'Relevant for CRA'.*	No *In the absence of information suggesting such effects being related to specific effects which*	Unknown for pesticide active substances *Overall no general MoAs for these effects.*	Yes *However, if these effects result only at the high dose and together with other overt signs of toxicity, there is less persuasive evidence of a direct neurotoxic effect.*	Decreased reactivity: Hyporeactivity, righting reflex (air drop), touch response (handling reactivity), approach response, pupil response, tail pinch response, analgesis reflex (nociception response), patellar reflex, etc.

	Hazard Identification (Step 1)							Hazard characterisation (Step 2)
Specific effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/ conclusions*	**Most appropriate indicator for effect** *(see Notes 1 and 3)*
					might be found when doing step 3.			Increased reactivity: Hyperreactivity, exaggerated auditory response (startle reflex), etc. Propioception: Propioception deficit, paraesthesia, hyperaesthesia, etc.
Autonomic division	No	Yes	Yes *The effects also occur in humans.*	In principle yes *See also Notes 1 and 3.*	No *In the absence of information suggesting such effects being related to specific effects which might be found when doing step 3.*	Unknown *No info in DTU or preliminary RIMV reports. Public literature to be screened. Overall no general MoAs for these effects with the exception of AChE inhibition by OPs and N-methylcarbamates (see Note 2).*	Yes *However, if these effects result only at the high dose and together with other overt signs of toxicity, there is less persuasive evidence of a direct neurotoxic effect.*	Miosis, mydriasis, salivation, lacrimation, urination, etc.
Neurochemical effects	No	Yes *Under*	Yes *The effects*	Yes	No *However,*	Known *Modulation of*	Yes *A statistically*	Brain AChE inhibition Erythrocyte AChE inhibition

	Hazard Identification (Step 1)							Hazard characterisation (Step 2)
Specific effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/ conclusions*	**Most appropriate indicator for effect** *(see Notes 1 and 3)*
		certain conditions; see column 'Relevant for CRA'.	*also occur in humans.*		*many pesticide classes induce oxidative stress that may lead to a decrease in erythrocyte AChE activity (see Note 5).*	*cholinergic transmission.*	*significant inhibition of brain, peripheral nerve or erythrocyte AChE ≥ 20% is considered toxicologically relevant ('adverse'). The inhibition of 20% may be considered with respect to the concurrent control group or with respect to the 'pre-exposure' values in the treated group.*	
Neuropathological effects	No	Yes	Yes	Yes	No	Unknown	Yes *Sciatic nerve axonopathy, without concurrent changes in motor neurons or spinal tracts, may be consistent with an increase of age-related effects due to systemic toxicity and diminished repair capacity of*	Axonal degeneration, myelin degeneration, neuronal degeneration/necrosis, dilated ventricles, etc.

	Hazard Identification (Step 1)							Hazard characterisation (Step 2)
Specific effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/ conclusions*	**Most appropriate indicator for effect** *(see Notes 1 and 3)*
							the nerve.	
Developmental neurotoxicity (DNT)	No	Yes	Yes	Yes	No *Current information is inadequate to assume that developmental effects at doses causing minimal maternal toxicity result only from maternal toxicity.*	Unknown	Yes *However, the high rate of proliferation and regeneration in the developing nervous system may lead to greater recovery or plasticity which could attenuate some injuries.*	Neuropathology (brain morphometry and weight), motor activity, auditory startle, behavioural ontogeny (righting reflex, swimming performance), learning and memory testing.

Notes:

1) Given the complexity of the nervous system that detects, integrates/interprets and responds to internal and external stimuli through nerves to effector organs (such as muscles and glands), the indicators assessed in neurotoxicology studies are not simple in nature and there are gaps of knowledge as to their physiological basis. This also precludes identification of the actual mode of action.

2) For organophosphates (OPs) and N-methylcarbamates, some motor, sensory and autonomic responses assessed by functional observational battery (FOB) may be based on acetylcholinesterase (AChE) inhibition. However, all these neurotoxic endpoints are considered together for risk assessment purposes.

3) A battery of functional tests, in contrast to a single test, is usually needed to evaluate the full complement of nervous system functions in animals.

4) Paraesthesia may be elicited by dermal exposure of either pyrethrins or pyrethroids, thus it can be considered as a local effect (ATSDR, 2003).

5) Since AChE is a membrane bound enzyme, oxidative stress induced by pesticide exposure may indirectly decrease AChE activity (Banerjee et al., 1999). Accordingly, AChE depression observed with pesticides other than OPs or N-methylcarbamates should be considered as an indirect effect.

6) The different specific effects relevant for inclusion of substances in CAGs have been shaded in different colours

7.2. Data collection for the nervous system (Step 3)

After identifying and characterizing the specific effects for the nervous system, a standardised and thorough review of relevant *in vivo* regulatory toxicity studies was performed for all pesticides considered relevant for CRA (as described in chapter 6.3: Data collection). The collection of toxicological data was performed by RIVM and EFSA (Appendix B) and includes only pesticides with an effect on the nervous system. The data collection contains information on 91 active substances, which are listed in Appendix H. A total of 68 active substances were identified by RIVM as having specific effects on the nervous system or as showing potential neurotoxic effects and 23 additional substances were identified by EFSA (24 if considering also Lufenuron, already included in the data collection performed by RIVM).

Specific considerations for the data collection for neurotoxicity are as follows:

Single and repeated dose studies were taken into consideration: for each specific effect, an acute or chronic NO(A)EL/ LO(A)EL was selected from either an acute or short/long-term experimental animal study.

In most cases, LD_{50} studies were not considered by RIVM but are listed by EFSA with additional information in the data collection regarding dose-related effects or possible mode of action (e.g. a consequence of severe systemic toxicity).

Doses provided in the DARs in ppm were converted to mg/kg bw/d using conversion factors recommended by the EFSA Scientific Committee (EFSA, 2012a).

7.3. CAGs for the nervous system (Step 4)

Two tables have been developed for grouping active substances, for acute and chronic effects on the nervous system (see Appendix C and D). Data were tabulated according to the specific effects on the nervous system, their relevant indicators, the active substance, its mode of action and the lowest experimental toxicity indices (NO(A)EL and LO(A)EL) were selected for each specific indicator of neurotoxicity. Considering the concurrence of effects occurring in an unpredicted manner in the nervous system, the specific effects were not ranked by a sequential order of indicators of effects. Sometimes the indices derived from several toxicological studies reported in the DARs.

Despite the fact that several non-specific effects were also described in the data collection, these effects were not considered for grouping. The same applied to those effects that occur after administration of high doses resulting in severe systemic toxicity. Also, substances that are used on non-edible plants were not considered for grouping.

7.3.1. Acute cumulative assessment groups for neurotoxicity

Of the 91 substances evaluated, 47 substances were included in the acute cumulative assessment group in which 45 substances showed effects on motor division, 20 on sensory division, and 29 on autonomic division. Some substances showed more than one effect and/or indicator and they were either observed at the same or similar NO(A)EL/LO(A)EL, possibly indicating a different sensitivity for the different effects.

7.3.2. Chronic cumulative assessment groups for neurotoxicity

Of the 91 substances evaluated, 64 substances were included in the chronic cumulative assessment groups in which 53 substances showed effects on the motor division, 21 showed effects on the sensory division and 24 on the autonomic division. In addition, for chronic cumulative assessment groups, neuropathological changes were also considered relevant. This is because some of the active substances after repeated exposure only showed neuropathological changes whereas for other substances, neuropathological changes were most sensitive. Neuropathological changes were observed

for 19 active substances. Some substances showed more than one effect and/or indicator and they were either observed at the same NO(A)EL/LO(A)EL or at a different NO(A)EL/LO(A)EL, possibly indicating a different sensitivity for the different effects.

The Panel is aware that the neurochemical changes represent a level of grouping for neurotoxic substances based on mechanism of action. However, AChE inhibitors play a prominent role in risk assessment and grouping based on neurochemical changes would result in an increased sensitivity for some substances. For this reason, and to keep consistency in the grouping approach, the neurochemical parameters should be used for further refinement when this mechanism of action is recognised. The number of substances included in CAGs for neurochemical changes were 13 and 15 for acute and chronic neurotoxicity, respectively. This type of grouping would benefit from further refinement when the mechanism of action is recognised and well understood.

8. Grouping methodology for the thyroid system

8.1. Hazard identification and characterisation for thyroid toxicity (Step 1 and 2)

Hazard identification and characterisation for thyroid were based on the information provided by the DTU Report (2012) and the open literature, and are systematically presented in table 2.

The thyroid functions as a gland that produces systemically acting hormones [calcitonin, thyroxin (T4) and triiodothyronine (T3)]. Specific effects were identified that concern the populations of calcitonin-producing parafollicular cells (C-cells) and thyroid hormone (T3/T4)-producing follicular cells. At least for the T3/T4 hormone system, changes in serum hormone levels may not only result from toxicity to the thyroid itself, but may result from modes of actions operating outside the thyroid (e.g. enzyme induction in the liver, peripheral metabolism of thyroid hormones). For data collection in step 3 of the methodology and grouping in step 4, it was therefore decided to consider not only effects concerning the thyroid gland itself, but also effects occurring at the thyroid hormone system level.

CAG level 1 (organ/organ systems level)

The most conservative level of grouping on a physiological basis comprised all substances affecting the thyroid hormone system (gland or hormones).

CAG level 2 (refinement on the phenomenological effect level)

Further sub-grouping was based on different general targets including the thyroid tissue (the follicular and the parafollicular cell population) and the associated hormone systems. Accordingly, distinction between two overall sub-groups (CAG2A and CAG2B) was recommended as explained in the following:

CAG2A: Substances affecting the thyroid parafollicular cells (C-cells) and/or calcitonin system

The parafollicular cells (C-cells) produce the hormone calcitonin which is involved in calcium homeostasis. While information on serum levels of calcitonin is generally not available from toxicological studies, two specific effects concerning the C-cells were identified:

- C-cell hyperplasia
- C-cell neoplasia

C-cell stimulation leading to hyperplasia is expected to play a promoting role in further progression to neoplasia, and hyperplasia and neoplasia are thus interrelated. It was therefore considered appropriate to combine substances displaying either or both of these effects into one level 2 CAG. In the absence of specific knowledge on the underlying modes of action leading to C-cell activation/proliferation of pesticide active substances, further sub-grouping (refinement based on mechanistic considerations) is currently not thought to be feasible.

CAG 2B: Substances affecting thyroid follicular cells and/or the thyroid hormone (T3/T4) system

The thyroid follicular cells produce the iodine-containing hormones called iodothyronines (thyroid hormones, TH), of which triiodothyronine (T3) and thyroxine (T4) are the most important. In adults, THs not only regulate energy metabolism, but also participate in regulation and maintenance of various physiological functions, while they are critical regulators of post-embryonic development. THs are essential for fetal and neonatal neurological development and developing children are particularly sensitive to perturbations in the thyroid system. Detailed considerations on the relevance of thyroid hormone system perturbation to humans have been included in Appendix E. In the context of CRA, the decrease in circulating TH levels or a decrease in TH action is regarded as a physiological alteration, which may lead to impairment of functional capacity, of general well-being (Lewis et al.,

2002) or development (depending on level, duration and timing of exposure to the relevant chemicals), and thus is considered as adverse.

The following specific effects were identified in animal studies for follicular cells or the T3/T4 system:

- Changes in serum T3 and/or T4 levels (generally in terms of decrease)
- Changes in serum TSH (generally in terms of increase)
- Follicular cell hypertrophy/hyperplasia and/or increased relative thyroid weight
- Follicular cell tumours

These specific effects are interrelated indicators for perturbations of the T3/T4 system. Typically a decrease in TH levels or antagonism of TH action is expected to result in various physiological and structural effects, observable in animal studies that represent a common toxicological pathway:

1. Perturbation of the thyroid hormone system via one or more mechanisms
2. Decrease in circulating thyroid hormone levels/impairment of thyroid hormone action
3. Compensatory increase in TSH
4. Stimulation of follicular cells
5. Follicular cell hyperplasia
6. Progression to follicular cell tumours

Although humans are assumed to be quantitatively less susceptible to development of follicular cell hyperplasia than e.g. rats, the specific effects listed above and observed in animal studies may be seen as important indicators for transient perturbation (transient TSH increase) or prolonged perturbation (prolonged TSH increase, follicular cell hyperplasia, follicular cell tumour formation) of the hypothalamic-pituitary-thyroid system, and were thus regarded to be relevant for CRA. Indicator effects, such as follicular cell hyperplasia and progression to follicular cell tumour may serve as a surrogate for one another for a perturbation of the thyroid hormone system, even in the absence of measurements of thyroid hormone (T3/T4, TSH) levels.

In addition to the specific effects used to define CAG2B and listed above, other effects were identified for some substances in the DARs. It was concluded that specific inflammation of the thyroid gland/lymphocytic thyroiditis, resulting in follicular cell degeneration, may be seen as one general mode of action that may impact thyroid function and may lead to changes in T3/T4 or TSH levels. Consequently, if inflammation/cell degeneration were clearly treatment-related and not attributable to aging alone, they should be considered together with the other listed specific effects in the screening of substances and for allocation to CAG2B.

Treatment-related pigmentation of follicular cells was not regarded as being relevant for grouping on its own, since it was not considered to be adverse as an isolated effect. Pigment deposition adversely affecting the thyroid would be expected to be accompanied by follicular cell degeneration and/or changes in T3/T4 or TSH levels and therefore would be covered by the other specific effects that would serve as indicators for inclusion in CAG2B.

Additional histopathological changes reported in DARs and listed in the DTU report, such as increase in iodine uptake, increased/decreased amount of colloid, small/large follicles, different shapes of follicular cells, increased vascularisation, increased vacuolisation, follicular cysts, follicular atrophy or necrosis of follicular cells, were regarded as being valid indicators for inclusion in a combined CAG2B. As these effects were concomitant with one or more of the specific effects identified in table 2, they were considered to be covered by these specific effects.

A number of observations concerning the thyroid were not considered to be relevant for grouping. These included amyloidosis in the mouse or congenital effects (thyroglossal duct cysts or ultimobranchial cysts resulting from persistence of embryonic structures (DTU, 2012; Frith et al.,

2000). Effects that were regarded as being age-related (e. g. mineralisation within follicular lumina) generally were not used for grouping. However, mineralisation in the follicle colloid that was considered to be treatment-related and to reflect premature aging of the thyroid was regarded to be relevant for grouping.

In summary, any of the following specific effects were recommended for data collection in step 3 and for allocation to the combined CAG2B in step 4: Changes in serum T3 or T4 levels, changes in serum TSH levels, follicular cell hypertrophy/hyperplasia and/or increased thyroid relative weight, follicular cell tumours. Thyroid inflammation/cell degeneration (and in some cases mineralisation in the follicule colloid) may be considered for allocation to the combined CAG2B group, but only if such effects have been established as substance-related and specific for the thyroid. Although other effects concerning the thyroid should be recorded in data collection in step 3, many of these effects would be covered by CAG2B and thus be reflected in one or more of the specific effects defining CAG2B.

Many substances affecting the thyroid follicular cells or the thyroid hormone system and thus allocated to CAG2B would be expected to generally be associated with a decrease in thyroid hormone (T3/T4) levels or ultimate thyroid hormone action. Additional modes of action contributing to the complexity of modulation of thyroid hormone action cannot be excluded. For example, some substances may affect deiodinase activity, and thus the extent of activation of T4 to T3 and inactivation to rT3, respectively. There may also be substances rather leading to thyroid stimulation or enhanced thyroid hormone release. If there is mechanistic information indicating substance-dependent direct stimulation of the thyroid or hyperthyroidism (increase in T3/T4, decrease in TSH), it might be considered at a later stage or in subsequent evaluation whether exclusion of the substance from CAG2B would appear feasible. Apart from such potential cases, a further refinement of CAG2B based on modes/mechanisms of action is currently not recommended by the Panel for the following reasons:

- For many pesticide active substances affecting the thyroid hormone system, the mode/mechanism of action has not been defined.
- In cases in which there is information on the mode/mechanism of action, it might be difficult to agree on the similarity or dissimilarity of various modes/mechanisms of action. For example, different substances might affect thyroid hormone clearance via enzyme induction (particularly in the liver), yet for individual substances this induction may be conveyed by different molecular pathways (activation of constitutive androstane receptor (CAR) versus pregnane X receptor (PXR)).
- Experimental studies involving rats indicated that, for mixtures of substances causing a decrease in T4 hormone levels via different individual mechanisms, the decrease in T4 levels may be predicted with a fair degree of accuracy by applying a dose addition model, especially for exposures occurring in the dose range below individual NOELs, while a response addition model would be more under-predictive (DTU, 2012; Crofton et al., 2005; Flippin et al., 2009).
- Some substances may act via several mechanisms, e.g. enzyme induction, displacement of endogenous hormone from plasma binding protein (Miller et al., 2009).
- Downstream organ or tissue effects will be based on the extent of interaction of the effector hormone with its cellular receptors. Different mechanisms may feed into the ultimate process of decrease/impairment of thyroid hormone action.

Table 2: Hazard identification/characterisation for thyroid toxicity

Hazard Identification (Step 1)								**Hazard characterisation (Step 2)**	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
Parafollicular cell (C-cell) hypertrophy and hyperplasia	No	Yes *Precursor effect for parafollicular cell neoplasia.*	Yes	Unknown	No	Unknown for pesticide active substances *Prolonged elevation of serum calcium levels can lead to stimulation of C-cell proliferation (Zabel, 1976).* *Some dithiocarbamates have been shown to induce rapid and sustained increases in intracellular calcium in PC12 cells (Sook Han et al., 2003), yet it is unknown whether this mechanism is relevant for stimulation of C-cells.* *Long-term activation of the glucagon-like-peptide-1 (GLP-1) receptor in rat C-cells may lead to C-cell activation and proliferation and eventually tumours*	Yes *To be grouped together with parafollicular cell (C-cell) neoplasia, since prolonged C-cell proliferation/hyperplasia may increase the risk for development of tumours.*	- C-cell hyperplasia - C-cell tumours (neoplasia/ adenoma) -C-cell tumours (neoplasia/ carcinoma)	C-cell hyperplasia seen as precursor effect for C-cell tumours.

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
						(Knudsen et al., 2010), yet it is unknown whether this mechanism may be relevant for pesticide active substances.			
Para-follicular cell (C-cell) neoplasia	No	Yes	Yes	Yes	No	Unknown for pesticide active substances *Chronic stimulation of C-cells leading to sustained proliferation is expected to increase the risk for tumours. Thus, mechanisms outlined above leading to C-cell hyperplasia may be operative.*	Yes *To be grouped together with parafollicular cell (C-cell) hyperplasia, since prolonged hyperplasia may increase the risk for development of tumours.*	- C-cell hyperplasia - C-cell tumours (neoplasia/ adenoma) -C-cell tumours (neoplasia/ carcinoma)	C-cell hyperplasia seen as precursor effect for C-cell tumours.
Changes in serum thyroid hormone (T3/T4) levels, generally in terms of decrease **(To be indicated**	No	Yes *Change in physiology that may lead to impairment of functional capacity,*	Yes *Although fluctuations in thyroid hormone levels may be less pronounced in humans*	Yes	No *Some MoAs affect the thyroid directly* *Other MoAs are*	One or more of the following MoAs may apply, although information on the MoA(s) for individual active substances is often not available: *Interference with iodide uptake into thyroid follicular cells via Na+/I-symporter.*	Yes *To be grouped within combined group CAG2B, together with substances eliciting a change in circulating TSH levels, thyroid follicular cell hypertrophy/hyperplasia /increased thyroid weight, or thyroid folli-*	-Decrease in circulating thyroid hormone (T3/T4) levels -compensatory increase in circulating TSH -stimulation of follicular cells	*All listed effects indicate perturbation of the thyroid hormone system or hypothalamic-pituitary-thyroid axis.*

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
during data collection whether decrease or increase)		*general well-being or development, depending on level, duration and timing of exposure.*	*(DTU, 2012).*		*indirect (displacement of hormones from plasma protein binding; enhancement of hormone metabolism via enzyme induction (mainly in the liver).*	*Inhibition of TH synthesis via inhibition of thyroperoxidase.* *Displacement of hormone from plasma protein binding.* *Enhancement of thyroid hormone metabolism/degradation, e.g. via enzyme induction (mainly in the liver).* *Inhibition of conversion of T4 to T3.* *Degeneration of thyroid follicular cells.* *(MoAs reviewed in DTU, 2012, or by Miller et al., 2009).*	*cular cell tumours, since these effects are interrelated.*	-follicular cell hyperplasia -follicular cell tumours	*While alterations in TSH may be transient or prolonged, follicular cell hyperplasia and tumour formation indicate prolonged stimulation via TSH / prolonged perturbation.*
Changes in circulating TSH, generally in terms of increase **(To be indi-**	No	*Response to decrease in circulating*	Yes *However, fluctuations in hormone levels may*	Yes	No *Response of hypothalamic-pituitary axis to*	Known MoA: *Compensatory response of the hypothalamic-pituitary axis to a decrease in circulating T3/T4 levels.*	Yes *To be grouped within combined group CAG2B, together with substances eliciting a change in circulating*	*As outlined above for changes in T3/T4 levels.*	*As outlined above for changes in T3/T4 levels.*

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
cated during data collection whether decrease or increase)		*T3/T4 levels or to impaired thyroid hormone action.*	*be less pronounced in humans (DTU, 2012).*		*change in circulating T3/T4 levels.* Direct response *if substance were to directly interfere with thyroid hormone action at hypothalamus or pituitary.*	Additional possible MoA (unknown whether these are relevant for pesticide active substances): *Impairment of thyroid hormone-dependent signalling within target cells of hypothalamus/pituitary, e.g. via antagonism towards thyroid hormone receptors/impairment of thyroid hormone-dependent transcriptional activation or inhibition of thyroid hormone uptake via membrane-situated transporters.*	*T3/T4 levels, thyroid follicular cell hypertrophy/hyperplasia or increased thyroid weight, or thyroid follicular cell tumours, since these effects are interrelated.* *Elevation of TSH can be considered to be a broader indicator of disruption of the thyroid hormone system in terms of underlying MoAs, since TSH levels would be expected to respond not only to changes in T3/T4 levels, but to changes in T3/T4 signalling.*		
Thyroid follicular cell hypertrophy/hyperplasia **and/or increased relative**	No	*Response to prolonged elevation of circulating TSH le-*	*Limited relevance; humans quantitatively less susceptible to follicu-*	Yes	No *Response to stimulation by TSH (not direct response of follicular*	Known MoA: *Response to stimulation by TSH.*	Yes *To be grouped within combined group CAG2B, together with substances eliciting a change in circulating T3/T4 levels, a change in TSH, or thyroid*	*As outlined above for changes in T3/T4 levels.*	*As outlined above for changes in T3/T4 levels.*

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
thyroid weight		*vels.*	*lar cell hyperplasia resulting from chemically-induced perturbation of thyroid hormone system than rats (Dellarco et al., 2006).*		*cells to chemical).*		*follicular cell tumours, since these effects are interrelated.*		
Thyroid follicular cell tumours	No	Yes	*Limited relevance; humans quantitatively less susceptible to follicular cell tumours resulting from chemically-induced*	Yes	No *Effect based on prolonged hyperplasia of follicular cells, due to prolonged stimulation via TSH.*	Known MoA: *Follicular cell hyperplasia may progress to follicular cell adenoma and carcinoma (Botts et al., 1991). Prolonged hyperplasia due to stimulation of follicular cells by TSH as a promoting factor for tumour formation.*	Yes *To be grouped within combined group CAG2B, together with substances eliciting a change in circulating T3/T4 levels, a change in TSH or follicular cell hyperplasia, since these effects are interrelated.*	*As outlined above for changes in T3/T4 levels.*	*As outlined above for changes in T3/T4 levels.*

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
			perturbation of thyroid hormone system than rats (Dellarco et al., 2006).						
Inflammation of thyroid gland (lymphocytic thyroiditis); **Degenerative changes in thyroid follicular cells**	No	Yes	Yes	No *Infiltrates of lymphocytes are sometimes observed in older rats and have been considered to be an incidental aging change (Frith et al., 2000), but some cases of lympho-*	Yes/No *may be specific or non-specific.*	Largely unknown for pesticide active substances *Immunomodulation has been described as a possible MoA for substance-related thyroiditis (Kitchen et al., 1979), yet it is unknown whether this MoA may be relevant for pesticide active substances. Degeneration via vacuolisation or alteration in lysosomal function may also be possible. For the pesticide spinosad, thyroid inflammation has been associated with*	Yes *For data collection, it may be helpful to consider thyroid inflammation and follicular cell degeneration as alerts for possible specific effects concerning thyroid function, if inflammation or cell degeneration are clearly substance-related. As inflammation and/or cell degeneration may be regarded as examples of general modes of action which may lead to changes in*	-Inflammation or lymphocytic thyroiditis -Degenerative changes in thyroid follicular cells *(As cells are damaged, there may be a transient increase in T3/T4 release, followed in the long run by a decline in T3/T4 hormone production and increase in TSH for compensa-*	*Thyroid inflammation and follicular cell degeneration can be seen as some general modes of action that may impact thyroid function, and thus may lead to specific effects in terms of changes in T3/T4 or TSH levels.*

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
				cytic thyroiditis/ inflammation or thyroid degeneration may be treatment-related (Kitchen et al., 1979; Yano et al., 2002).		*vacuolisation, which in this case appears to correspond to lysosomal phospholipidosis (Yano et al., 2002).*	*T3/T4 or TSH levels, inflammation/cell degeneration should be included among the parameters for defining the merged group CAG2B.*	*tion).*	
Pigmentation within thyroid follicular cells or follicular lumina	No	No *Not adverse as an isolated effect, but may in some cases be associated with disturbed thyroid function.*	Yes	No *Pigmentation may be observed in the context of normal aging, and/or be treatment-related (Frith et al., 2000; Tajima et al., 1985). The*	Yes/No *May be specific or non-specific.*	Unknown for pesticide active substances *Deposition of pigment consisting of the active substance or metabolites indicates that mechanisms leading to concentration of the substance or its metabolites within follicular cells may be operative. However, the identity of the pigment is usually not specified.*	No *Pigmentation alone is not regarded as a sufficient basis for grouping, but in some cases may indicate accumulation of test substance or metabolites in the thyroid.* *Pigment deposition adversely affecting the thyroid would be reflected by changes in thyroid function. In this case, such substances would be covered by the merged group*		

Hazard Identification (Step 1)								Hazard characterisation (Step 2)	
Specific Effect	**Local** Yes/No *Remarks*	**Adverse** Yes/No *Remarks*	**Relevant for humans** Yes/No *Remarks*	**Unambiguous unique** Yes/No *Remarks*	**Non-specific** Yes/No *Remarks*	**MoA** Known/ Presumed/ Unknown *Remarks*	**Relevant for CRA** Yes/No *Overall remarks/conclusions*	**Indicators in sequence of occurence** *Remarks*	**Most appropriate indicator for effect** *Remarks*
				identity of pigment is usually not specified.			*CAG2B for substances affecting the thyroid follicular cells and/or the thyroid hormone system.*		
Mine-ralisation, clumps of minerals observed within lumen of follicles	No	No *Minerali sation occurs in adult rats and is consider ed a normal aging change (Frith et al., 2000).*	Unknown	Unknown	Unknown	Unknown	No *Unless mineralisation of the follicular colloid is regarded to be a substance-related effect reflecting premature aging of the thyroid*		

Notes:
Taking into account the information provided by the DTU Report (DTU, 2012) and the open literature, the following recommendations were made for grouping:

CAG level 1 (organ/systems level)

CAG level 1 would comprise all substances affecting the thyroid system (the thyroid, T3/T4 and calcitonin systems).

CAG level 2 (refinement on the phenomenological level)

CAG2A: Substances affecting the thyroid parafollicular cells (C-cells) and/or calcitonin system (effects in yellow)

Two specific effects concerning the C-cells were identified: C-cell hyperplasia and C-cell neoplasia. Since C-cell stimulation leading to hyperplasia is expected to play a promoting role in further progression to neoplasia, it was proposed to combine substances displaying either or both of these effects into one level 2 CAG.

Specific effects combined into one CAG2 level for grouping were shaded in yellow.

CAG 2B: Substances affecting the thyroid follicular cells and/or the thyroid hormone system (effects in green)

It is thought useful to combine substances eliciting any of the following specific effects into one common level 2 CAG, as they are regarded as being interrelated and connected to one another by a chain of events:

- Changes in serum T3 and/or T4 levels (generally in terms of decrease)
- Changes in serum TSH (generally in terms of increase)
- Follicular cell hypertrophy/hyperplasia and/or increased relative thyroid weight
- Follicular cell tumours

Specific effects combined into one CAG 2 level for grouping were shaded in green.

In addition to the specific unambiguous effects listed above for defining CAG2B, other effects were identified in the DARs:
It was concluded that inflammation of the thyroid gland/lymphocytic thyroiditis, resulting in follicular cell degeneration, may be seen as one general mode of action that may impact thyroid function and may lead to changes in T3/T4 or TSH levels. Consequently, if inflammation/cell degeneration were clearly treatment-related and not attributable to aging alone, they could be considered together with the other listed specific effects in the screening of substances and for allocation to CAG2B.

Treatment-related pigmentation of follicular cells was not regarded as being relevant for grouping on its own, since it was not considered to be adverse as an isolated effect. Pigment deposition adversely affecting the thyroid would be expected to be accompanied by follicular cell degeneration and/or changes in T3/T4 or TSH levels and therefore would be covered by the other specific effects employed for allocation to CAG2B. Additional histopathological changes reported in DARs and listed in the DTU report, such as increased/decreased amount of colloids, small/large follicles, different shapes of follicular cells, increased vascularisation, increased vacuolisation, follicular cysts, follicular atrophy or necrosis of follicular cells were regarded as being indicators for inclusion in the combined CAG2B.

A number of observations concerning the thyroid were not considered to be relevant for grouping. These included amyloidosis in the mouse and congenital effects (thyroglossal duct cysts or ultimobranchial cysts resulting from persistence of embryonic structures (DTU, 2012; Frith et al., 2000). Effects that were regarded as age-related (e. g. mineralisation within follicular lumina) were generally not used for grouping, although mineralisation in follicle colloid was considered relevant for grouping if it reflected substance-related premature aging of the thyroid.

8.2. Data collection for the thyroid system (Step 3)

After identifying and characterizing the specific effects for thyroid toxicity, relevant toxicological data was screened for each pesticide (considered relevant for CRA as described in chapter 6.3: Data collection) through a standardised and thorough review of relevant *in vivo* regulatory toxicity studies. The collection of toxicological data was performed by EFSA (Appendix F) and includes only pesticides causing effects on the thyroid system. The data collection contains information on 113 active substances, which are listed in Appendix H.

Specific considerations for the data collection for thyroid toxicity were as follows:

Only NO(A)ELs/LO(A)ELs from repeated dose studies were considered with regard to the specific effects since the thyroid is not being investigated and/or generally less likely to be observed in acute toxicity studies.

While substances affecting the thyroid system were generally identified via the specific effects established under chapter 8.1, any additional effects were recorded and subsequently scrutinised with respect to relevance for grouping. Data were checked to confirm that effects were specific to the thyroid. For example, an increase in relative thyroid weight occurring in isolation without other reported thyroid effects was only regarded as being specific if this was not the result of generalised toxicity, e.g. not associated with body weight decrease.

Doses provided in the DARs in ppm were converted to mg/kg bw/d using conversion factors recommended by the EFSA Scientific Committee 2012 (EFSA, 2012a).

8.3. CAGs for the thyroid system (Step 4)

Grouping of substances affecting the thyroid system was performed for substances retrieved during the data collection (Appendix F). The result of grouping is shown in Appendix G, in which individual substances are listed in relation to CAGs, along with their most sensitive NOAELs/LOAELs for the specific effects. Very often, more than one study and more than one specific effect within the data collection supported assignment to a sub-group. In such cases, only the most sensitive effect occurring in the most sensitive species was listed in the Appendix. In addition, when available, information on presumed or known modes/mechanisms of action was provided.

CAG1

Of the 113 active substances in the database, 101 substances were found to have an effect on the thyroid system. Accordingly, these substances were allocated to CAG1 as the most conservative level of grouping.

Considering the specific effects identified and characterised in chapter 8.1., substances from CAG1 were sub-grouped to CAG2A and CAG2B.

CAG2A

In total, 22 substances displayed one or more of the indicators for substances affecting the thyroid parafollicular cells (C-cells), namely C-cell hyperplasia or C-cell neoplasia (C-cell tumours, C-cell adenoma, C-cell carcinoma), and were consequently allocated to CAG2A.

CAG2B

96 substances were identified as substances affecting the thyroid follicular cells and/or the thyroid hormone (T3/T4) system, and allocated to CAG2B. As outlined in chapter 8.1. some effects were described in the DARs, such as changes in size of follicles, changes in colloids, different shapes of follicular cells, or increased iodide uptake, that generally were concomitant with one or more of the

specific effects identified in table 2, and were thus considered to be covered by the combined group CAG2B.

Comparing the two sub-groups CAG2A and CAG2B, it was noted that 17 of the 22 substances in CAG2A were also grouped into CAG2B (and thus displayed effects on the parafollicular C-cells as well as on the follicular cells or T3/T4 system), while for the other 5 substances of the CAG2A group, effects were only reported on the parafollicular cells. Thus, it appears that at least part of CAG2A might be covered by CAG2B. Evidence has been provided in the literature suggesting a functional relationship between parafollicular cells and follicular cells or the hypothalamic-pituitary-thyroid axis. For example, C-cells express the TSH receptor (Morillo-Bernal et al., 2009). However, the underlying mechanisms relevant for the induction of C-cell hyperplasia by pesticide active substances remain to be defined. In particular, it needs to be elucidated whether mechanisms affecting the C-cells alone may be distinguished from mechanisms linking both C-cell and follicular cell hyperplasia.

In conclusion, two sub-groups are currently recommended for CRA for substances affecting the thyroid system. These CAGs are defined by effects related to the different cell populations within the thyroid system. This grouping may be subject to revision as new mechanistic data on the mode/mechanism of action of individual substances and on the functional interaction between the two cell types in the thyroid become available.

9. Considerations of potency during conduct of CRA

Pesticides may cause toxic effects at multiple sites by a single (e.g. neurotoxic) mode of action. Therefore, substances can be grouped in more than one CAG. The effects considered for the establishment of reference values (i.e. Acceptable Daily Intake (ADI) and Acute Reference Dose (ARfD)) are not necessarily representative for the CAGs, i.e. an effect observed at higher dose levels may be the specific effect relevant for grouping. Reference values for the specific effects within the different CAGs and index compounds (IC) may be established, but such considerations are outside the scope of the present Opinion on developing a general methodology. This would represent a future step and would require the identification of IC within each CAG (see EFSA, 2008, 2009).

For each active substance within a CAG, the difference between its critical NOAEL (used in the regulatory context for reference value setting) and its NOAEL for a specific effect should be considered. For instance, the contribution of an active substance to the cumulative toxicity in a CAG will be inversely proportional to the difference in values between its NOAEL for the specific effect and its critical NOAEL. That is, if the difference between these NOAELs is small the contribution of a substance to the cumulative toxicity in a CAG will be higher than the contribution of a substance for which the difference between its NOAELs is large.

As a consequence, if for example the critical NOAEL is 5-fold lower than the NOAEL for a specific effect (assuming an uncertainty factor of typically 100 and that the exposure to the pesticide is 100% of its ADI), the hazard quotient would be 0.20 for the specific effect (HQ = exposure*100/NOAEL). The pesticide of concern would contribute at most with 20% to the total cumulative exposure. Combined exposure to other compounds sharing the specific effect would cease to be acceptable when the hazard index exceeds 1.0. Whilst this is relatively modest given the uncertainties in such a risk assessment, one might still argue that the margin of difference, i.e. 5-fold, is insufficient.

Risk managers should therefore decide as to what would be an appropriate margin of difference between the critical NOAEL and the NOAEL for a specific effect. It is important to note that without clear criteria, all compounds of a CAG will have to be included in the respective CRA, which would involve substantial resources, which could be argued to be unjustified. For a transparent approach, clear criteria are needed for an efficient use of CAGs, e.g. excluding substances with a very low hazard quotient for the specific effect or those with a NOAEL for specific effects close to the maximum dose tested (e.g. tetramethrin and sumithrin that were not included in a common assessment group in the US EPA assessment because low-hazard pyrethroids do not induce typical neurobehavioral effects at the highest dose tested, as noted in chapter 3.3).

10. Uncertainties and limitations

The Panel was tasked with developing a methodology for grouping pesticide active substances into CAGs based on their common or shared toxic effects for their use in CRA. In the absence of experimental information about their actual combination effects, the combination effects of the individual pesticides had to be anticipated on the basis of their toxicological profiles. This is a challenging task that stretches the limits of current scientific knowledge.

The available evidence with experimentally tested mixtures (Kortenkamp et al., 2009) shows that combination effects with substances that act via similar mechanisms and modes of action are highly likely, all the more so when the mixture components have a common molecular target, such as inhibition of the same enzyme by an identical mechanism. Accordingly, chemicals with common structural features are likely to interact with the same molecular targets, and can be expected to produce combination effects after simultaneous exposures. The certainty with which combination effects can be predicted in such cases is quite high, but this comes at the price of potentially underestimating risks by ignoring chemicals that might also contribute to a joint response by acting further upstream or downstream on an effector chain or through different pathways (US NRC, 2008).

Conversely, the uncertainties associated with predicting possible combination effects increase the more the assessments are based on effects at a level of biological complexity rather than on molecular mechanism of action. This uncertainty has to be balanced against the higher degree of protection that is afforded by considering a wider range of chemicals.

The Panel recognises that the fundamental difficulties in grouping pesticides based on wide inclusion criteria can only be resolved in the foreseeable future by obtaining better information on the modes of action for each of the toxic effects caused by the substances. Substantial refinements of CAGs may be achieved through increased knowledge about the molecular/cellular mechanisms of actions shared by groups of pesticides and about the link between mechanisms and effects at the organ and organism level.

Until such information becomes available, the approach proposed by the Panel represents what is achievable based on current scientific data. It is the Panel's view that the alternative option of using quite narrow grouping criteria derived from molecular mechanisms and modes of action is likely to capture only a fraction of the potential cumulative risks. This view is supported by empirical evidence on mixtures composed of multiple chemicals that operate through a variety of mechanisms.

The Panel is aware that information included in the proposed CAGs was only derived from DARs provided by the rapporteur member states and not the raw data that underpin the DARs. Considerable uncertainties arise from the fact that DARs contain different levels of details on the toxicological assessments of the raw data. Also, considerable changes and developments have occurred with regard to data requirements and study protocols (e.g. multigeneration study, 28 day study, DNT study) that consequently have an impact on the uncertainties associated with the CAGs proposed in this opinion. Furthermore, some endpoints considered important today have not been reported in older DARs and consequently the lack of information about certain effects may result in the possibility that some substances are not included in certain CAGs. Inconsistent terminology in the DARs is also a source of uncertainty, in particular in relation to descriptions of histopathological and developmental findings.

General uncertainties affecting CRA have carefully been assessed in the previous PPR Opinion (EFSA, 2009).

11. Conclusions

Following a mandate received by EFSA, the PPR Panel has developed a scientific Opinion on the identification of pesticides to be included in CAGs on the basis of their toxicological profile. Consequently, such pesticides have been identified in the present opinion.

The Opinion does neither present considerations on exposure nor a proposal for an overall framework for CRA or considerations on compounds not regulated under the Regulation (EC) 1107/2009[13] as such proposals and considerations would not be within the scope of the present mandate.

The evaluation performed by the PPR Panel is not intended to challenge peer-reviewed and agreed reference values and/or points of departure (NOAEL, LOAEL).

The Opinion rather describes a CAG methodology, specifically developed for pesticides, based on common toxic effects, defining a general approach and criteria for the grouping of pesticides for CRA. Cumulative assessment groups of pesticides were provided for the nervous system and the thyroid system. The Panel has taken into account previous PPR Panel opinions on methodologies for the assessment of cumulative and synergistic risks from pesticides to human health and on triazole fungicides as a working example of grouping pesticides based on structure and pesticidal mode of action. The present methodology was developed on the basis of oral toxicity studies in order to take cumulative effects into account in the decision on application concerning MRLs. Although this methodology was specifically developed for dietary exposures to pesticide residues, it could in principle be applied to non-dietary exposures (i.e. operator, worker, bystander and resident exposure) for CRA.

The proposed grouping approach in this Opinion followed directions from the European Commission and was based on shared patterns of toxicologically relevant and unambiguously defined effects that occur at the level of tissues, organs and physiological systems. The basis for the grouping methodology assumed that pesticides producing the same toxic effects in tissues, organs and physiological systems have the capability of producing joint, cumulative toxicity. This represented a significant challenge, considering that evaluations for potential combined effects have to be made with very limited experimental data about cumulative toxicity. The Panel concludes that this grouping approach is relevant and scientifically justified in view of evidence that chemically unrelated substances may produce common toxicity in target organs, tissues or endpoints (e.g. developmental and reproductive end-points) even without a common mode of action. The PPR Panel envisages that further refinements based on common mechanisms of toxicity could be developed when information on biochemical effects becomes available for more pesticide active substances. However, any information that justifies deviation from dose-addition might also be necessary to consider. Based on current knowledge, synergistic interactions are not expected to occur at the low exposure levels that are typical of dietary pesticide residues. Accordingly, the PPR Panel considers that the proposed CAGs should be used in the context of MRL setting and risk assessment assuming mainly dose additive combination effects.

Although the data collection is confined to EU approved pesticides in this opinion, in principle, all pesticides that can occur as residues may be considered for CRA based on CAGs as defined according to the approach laid down in this opinion. The data collection for establishment of CAGs was based on a standardized and thorough review of DARs although preferably all available and relevant information (e.g. literature, non EU governmental reports) should also be taken into account. For the present Opinion all active substances approved in the EU until 1st January 2012 have been considered.

[13] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EC and 91/414/EEC. Official Journal L 309, 1-50. 24 November 2009.

In addition, the PPR Panel acknowledges that, based on currently available data sets, non-authorised pesticides identified as residues in the EU food commodities cannot be included in CAGs; however, their inclusion may be considered as a further development of CRA.

The methodology in the current Opinion comprises four main steps:

- Identification of the specific effects as follows:

  i) exclusion of local effects
  ii) exclusion of non-adverse effects
  iii) exclusion of effects not relevant to humans
  iv) evaluation of the unambiguous nature of the effect
  v) identification of non-specific effects

- Characterisation of the specific effects
- Data collection
- Grouping of pesticides into cumulative assessment groups

The CAGs for neurotoxic substances were based on results from acute and chronic (repeated-dose) experimental studies included in the available DARs. The specific effects were mainly obtained from Functional Observation Batteries (FOBs) and thus they were restricted to the motor, sensory and autonomic divisions of the nervous system. For some active substances, neurochemical indicators of effects were also available, particularly erythrocyte or brain AChE activity. Accordingly, four principal groupings were characterized, one for each of the above-mentioned functional divisions of the nervous system and another CAG for neurochemical indicators. The latter should be considered as a further refinement for grouping because it's based on the toxic mechanism of action. Moreover, CAGs were developed for acute and chronic exposure scenarios, with the latter also including neuropathological changes as a distinct and additional CAG since acute exposures to pesticides are not expected to induce adverse effects at a histopathological level.

The CAGs for thyroid effects have balanced the presence of different targets within the hypothalamic-pituitary-thyroid axis, and the limitations of data on mode of actions, as well as the potential for different mechanisms of action to result in a common effect, e.g. reduced T3/T4 level. Thus, two principal groupings were characterized (substances affecting the thyroid follicular cells and the T3/T4 system, and those affecting the C-cells/calcitonin system). As a further refinement, substances eliciting hyperthyroidism might be identified with the support of expert judgement. Thyroids effects resulting from acute exposure were not observed in the DARs, and are regarded to be less likely to occur than effects following repeated exposure (OECD Guidance on ARfD setting, 2010).

Application of this methodology has yielded CAGs with sometimes large numbers of pesticides (see below). Although some CAGs contain a large number of pesticides, littlei ndication of cumulative risk may be inferred from the size of CAGs per. Even with large CAGs, it is possible that the majority of pesticides contribute little to a combination effect, either because exposure is very low, or because potency in relation to the effect considered is weak. Instead, cumulative effects are likely to be driven mainly by a few active substances.

Preliminary work already performed on several target organs/tissues (adrenal, eye, liver, reproductive system) should be continued in order to achieve a timely advance of CAG application to these other organs/systems. The PPR Panel also recognizes that the CAGs based on common toxic effects can be changed by adding more information on pesticide mode of actions and toxicity pathways and with a better understanding about the relevance of dissimilar modes of action for common toxic effects. Moreover, information that justifies any deviation from dose addition must be considered for such a refinement. These issues will be explored further by the Panel within its mandate on the relevance on

dissimilar mode of action. Most importantly, CAGs should be updated by applying a consistent methodology.

EFSA intends to gradually implement CRA in the framework of EU regulations on pesticides by first addressing the cumulative risk related to the CAGs established in this opinion. In this context, EFSA will consult the European Commission and Member States to guarantee coherence between risk assessment and risk management in the conduct of CRA.

On uncertainties the PPR Panel acknowledges that quantification of the uncertainties is complex and that specific guidance for uncertainty analysis regarding hazard and risk characterisation for multiple chemicals should be developed.

12. Recommendations

The PPR Panel makes several recommendations for the implementation of this CAG methodology in CRA to support MRL setting:

- The implementation of the methodology based on specific effects should be supported by expert judgement, in order to identify the effects relevant for grouping according to the criteria laid down in the opinion.
- When performing cumulative risk assessment, exposure and potency considerations should be taken into account by risk assessors to make best use of resources.
- The external scientific reports on data collection for specific endpoints (DTU, 2012; ANSES/ICPS/RIVM, 2013) and preliminary work of EFSA, already carried out for relevant target organs and tissues should be used as a starting point for future groupings of pesticides for effects in organs/organ systems other than the thyroid and the nervous system.
- The IPCS framework on CRA (IPCS, 2009) should be applied to assess and report information on mode of action in future DARs. Further consideration should then be given to the inclusion of more mechanistic studies and new methodologies within the data requirements for pesticides.
- Guidance for the implementation of CAG methodology in the AIR (Annex I renewal) programme should be developed by EFSA and tailored to the different programmes to support MRL setting.
- The CAGs, established following the methodology developed in the current opinion, could also be used for non-dietary CRA.
- All pesticides covered by Regulation (EC) No 396/2005 (including import tolerances) should be considered for inclusion in CAGs. In order to do so, other sources of information than EU peer-reviewed data should be considered (e.g. JMPR, US EPA).
- A procedure should be developed to update the CAGs for the inclusion of new active substances.
- Specific guidance for uncertainty analysis regarding hazard identification and characterisation for multiple chemicals should be developed.
- The applicability of the “exclusion” approach could be explored when dealing with substances other than pesticides under the EFSA remit.

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GLOSSARY

Acute toxicity: Adverse effects of finite duration occurring within a short time (up to 14 d) after administration of a single dose (or exposure to a given concentration) of a test substance or after multiple doses (exposures), usually within 24 h of a starting point (which may be exposure to the toxicant, or loss of reserve capacity, or developmental change, etc.).

Additive effect: Effect observed after exposure to two or more chemical agents which act jointly but do not interact. The total effect is the simple sum of the effects of separate exposure to the agents under the same conditions.

ADI (Acceptable daily intake): Estimate of the amount of substance in food expressed on a body weight basis, that can be ingested daily over a lifetime, without appreciable risk to any consumer on the basis of all known facts at the time of evaluation, taking into account sensitive groups within the population (e.g. children and the unborn).

Adverse effect: Change in the morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences

Aggregate risk: Risk associated with all pathways and routes of exposure to a single chemical.

AIR (Annex I Renewal): Renewal of active substances included in Annex I of Council Directive 91/414/EEC.

ARfD (Acute reference dose): Estimate of the amount of substance in food and/or drinking water, expressed on a body weight basis, that can be ingested over a short period of time, usually during one day, without appreciable risk to the consumer on the basis of the data produced by appropriate studies and taking into account sensitive groups within the population (e.g. children and the unborn).

Chronic effect: Consequence that develops slowly and/or has a long lasting course: may be applied to an effect that develops rapidly and is long-lasting.

Combined toxicity: Response of a biological system to several chemicals, either after simultaneous or sequential exposure. It can take three possible forms: dose-addition, response-addition or interaction. In the context of this opinion, only dose-addition is considered.

Common mechanism group: Group of chemicals determined to cause a common toxic effect by a common mechanism of toxicity

Common mechanism of toxicity: Pertains to two or more substances that cause a common toxic effect to experimental animals or to human health by the same, or essentially the same, sequence of major biochemical events. Hence, the underlying basis of the toxicity is the same, or essentially the same, for each chemical.

Common toxic effect: Two or more substances that are known to cause the same toxic effect in or at the same anatomical or physiological site or location (e.g. same organ or tissue). Thus, a toxic effect observed in studies involving animals exposed to a pesticide is considered common with a toxic effect caused by another chemical if there is concordance with both site and nature of the effect.

Critical NOAEL: No Observed Adverse Effect Level (NOAEL) for the effect upon which the ADI (or ARfD) is based.

Cumulative assessment group (CAG): Group of active substances that could plausibly act by a common mode of action, not all of which will necessarily do so. The first and most conservative level

of grouping is based on the organ or organ system level being the target of the pesticide toxicity (CAG 1). Further refinement to form a second level of grouping (CAG 2) is based on the identification and characterisation of specific effects in the organ or organ system. (Due to shortage of information on underlying mechanism/mode of action for many pesticides, further sub-grouping as performed in the DTU report (CAG 3 and CAG 4) are not considered relevant to this opinion.)

Cumulative risk: In the context of this opinion, it corresponds to the risk resulting from exposure to more than one pesticidal active substance via the diet.

Dissimilar mode of action: Occurs when the mode of action and possibly, but not necessarily, the nature and sites of toxic effects differ between the chemicals in a mixture, and one chemical does not influence the toxicity of another. The effects of exposure to such a mixture are the combination of the effects of each component compound (also referred to as response-addition).

Dose addition: see similar mode of action.

Exclusion approach: Establishment of CAGs on basis of common toxicological effects even in the absence of a clearly established (common) mode of action. A compound is excluded from a CAG only when it can be shown that it does not exhibit the common toxicological effect.

Hazard: Inherent property of an agent (e.g. pesticide) or situation having the potential to cause adverse effects when an organism, system, or (sub-) population is exposed to that agent or situation.

Hazard assessment: Process that includes hazard identification and characterisation and focuses on the hazard in contrast to risk assessment where exposure assessment is a distinct additional step.

Hazard characterisation: Qualitative and, wherever possible, quantitative description of the inherent property of an agent or situation having the potential to cause adverse effects. This is the second stage in the process of hazard assessment.

Hazard identification: Identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system, or (sub) population. This is the first stage in the process of hazard assessment.

Hazard index (HI): Sum of Hazard Quotients, i.e. ratios between exposure and the reference value for the common toxic effect of each component in the CAG.

Inclusion approach: Establishment of CAGs on basis of common toxicological effects only if a common mode of action is clearly established.

Index compound (IC): The chemical used as the point of reference for standardizing the common toxicity of the chemical members of the CAG. The index compound should have a clearly defined dose-response, be well defined for the common mechanism of toxicity, and have a toxicological/biological profile for the common toxicity that is representative of the CAG.

Indicator: In the context of this opinion, an observed or measured effect relevant for grouping which is interrelated or linked to one or more specific adverse effects, either by representing a different outcome of a common toxicological pathway or a different step within a chain of events. The indicator may thus serve as a surrogate for other related effects that have not been determined or measured in a particular study. For example, an increase in circulating TSH levels or thyroid follicular cell hypertrophy would be expected to be related to a low circulating thyroid hormone (T3/T4) levels and would thus be regarded as indicative of disruption of the thyroid hormone system, even in the absence of thyroid hormone measurements.

Interaction: Umbrella term for synergies (mixture effects greater than expected) and antagonisms (mixture effects smaller than expected). Interactions can be judged in relation to additivity expectations derived from dose addition or independent action

Maximum residue level (MRL): Upper legal level of a concentration for a pesticide residue in or on food or feed set in accordance with Regulation 396/2005, based on good agricultural practice and the lowest consumer exposure necessary to protect vulnerable consumers.

Mechanism of action: Detailed explanation of the individual biochemical and physiological events leading to a toxic effect.

Mechanism of toxicity: Mechanism of toxicity is defined as the steps leading to a toxic effect following interaction of a pesticide with biological targets. All steps leading to an effect do not need to be specifically understood. Rather, it is the identification of the crucial events following chemical interaction that are required to describe a mechanism of toxicity. In the context of this document, mechanism of toxicity refers to the mechanism by which a pesticide is toxic to humans or experimental animals, and not the mechanism by which it is toxic to target or intended species (i.e. its mechanism of pesticidal action). With some pesticides, however, the mechanism responsible for causing toxicity to humans or experimental animals is similar to the mechanism of pesticidal action.

Mode of action (MoA): Biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. It refers to the major steps leading to an adverse health effect following interaction of the compound with biological targets, it does not imply full understanding of mechanism of action at the molecular level.

NOAEL/LOAEL (No Observed Adverse Effect Level / Lowest Observed Adverse Effect Level): In general, effects that are non-adverse do not qualify to be the basis for the setting of neither a NOAEL nor a LOAEL (JMPR, 2006). However they might be considered as specific effects for CAGs. The decision to consider an effect as non-adverse but relevant for a CAG needs to be supported by expert judgement.

NOEL/LOEL: All the findings (endpoints) that were indicated in the outsourced contract as indicative for those effects have been reported for each substance, with their respective NOEL/LOELs. The selection of NOELs and LOELs was performed, as requested by EFSA, without any interpretation on whether an effect is to be considered adverse or not adverse.

Phenomenological effect: In the context of this Opinion corresponds to a range of effects, morphological and/or functional, that can be observed in a target organ or organ system. These effects could also represent a continuum of pathological findings.

Pesticide formulations, plant protection products (PPP): Mixtures or solutions composed of two or more active substances intended for use as a plant protection product or as an adjuvant.

Rapporteur member state (RMS): Member State which undertakes the task of evaluating the dossier of an active substance, safener or synergist.

Residues: Are consisting of one or more substances present in or on plants or plant products, edible animal products, drinking water or elsewhere in the environment and resulting from the use of a plant protection product, including their metabolites, breakdown or reaction products.

Response addition: see dissimilar mode of action

Risk assessment: Process intended to calculate or estimate the risk to a given target organism, system, or (sub-) population, including the identification of attendant uncertainties, following exposure to a

particular pesticide or agent of concern as well as the characteristics of the specific target system. It is the first component in a risk analysis process.

Similar mode of action: Describes the mode of action when all chemicals in the mixture act by the same mechanism/mode of action, and differ only in their potencies. The effects of exposure to a mixture of these compounds are assumed to be the sum of the potency-corrected effects of each component (also referred to as dose-addition).

Specific effect: In the context of this opinion, a specific effect is a well characterized and unambiguous toxicological effect relevant for grouping in the context of cumulative risk assessment. For sake of clarity, a specific effect in this Opinion should also be considered in the context of the different level of organisation of the nervous system (i.e. effects on motor division, effects on sensory division, and effects on autonomic division).

Substances: Chemical elements and compounds, as they occur naturally or by manufacture, including any impurity inevitably resulting from the manufacturing process. Other synonyms are used in this opinion: active substances, pesticides.

Synergism: Pharmacological or toxicological interaction in which the combined biological effect of two or more substances is greater than expected on the basis of the simple summation of the toxicity of each of the individual substances.

Toxic effect: Effect known (or can reasonably be expected) to occur in experimental animals and presumably in humans that results from exposure to a chemical substance and that will or can reasonably be expected to endanger or adversely affect the quality of life.

APPENDICES

A. BACKGROUND FOR HAZARD IDENTIFICATION AND CHARACTERISATION FOR EFFECTS ON THE NERVOUS SYSTEM

1. A methodological approach to neurotoxicity risk assessment[14]

The nervous system is an extremely complex entity and closely related to other organ systems, especially the immune and endocrine systems. A number of major anatomic and functional divisions can be identified as potential targets for adverse effects when exposed to toxic substances. The anatomic divisions include both the central and the peripheral nervous system. The central nervous system consists of the brain and spinal cord, while the peripheral nervous system includes both afferent and efferent nerve pathways. Afferent nerves carry sensory information from peripheral receptors toward the brain while efferent nerves carry motor commands from the brain to effector organs (muscles or glands). Efferent pathways can in turn be subdivided into somatic nerves, that carry motor information to skeletal muscles, and autonomic nerves that carry motor information to cardiac muscle, smooth muscles and exocrine glands. Accordingly, motor, sensory and autonomic functions are considered as functional divisions of the nervous system. The specialised functions of the nervous system make this organ system vulnerable to many toxic compounds that may affect multiple targets in different ways. This is the case with insecticides, pesticides designed to control insects by interfering with the nervous system of selected target organisms.

Neurotoxicity can be measured at multiple levels of organization. Structural neurotoxic effects include neuroanatomical abnormalities occurring at any level of nervous system organisation. In turn, functional changes encompass neurochemical, neurophysiological or behavioural effects leading to alterations in motor, sensory, autonomic and cognitive function.

When identifying a substance as being neurotoxic, it is important to ascertain whether the nervous system is the primary target organ of the substance. The type, severity, number and reversibility of the effect should also be considered. Irreversible neurotoxic effects are of high concern and usually involve structural changes or long lasting functional effects. Transient overt effects do not, by themselves, exclude the possibility of a permanent lesion has occurred. In contrast to other tissues, nerve cells show limited ability to replace or regenerate neural damage. Thus, when cell death has occurred, the lack of potential of neurons to achieve full recovery can result in permanent disruption and damage of the nervous system. Although the large reserve capacity of this organ system may compensate for the damage, the resulting reduction in the reserve capacity should be regarded as an adverse effect. Compensation may be suspected when a neurotoxic effect slowly resolves during the life span.

The uncertainties associated with data from any neurotoxic indicator can often be greatly reduced if interpreted within the context of other neurotoxicological measures and systemic toxicity indicators, particularly if such measures are taken concurrently. Studies that contain results from only one type of indicator can often be very difficult to interpret; hence multiple measures provide a multidimensional approach that allows for a better interpretation of effects. However, neurotoxic effects that are secondary to systemic toxicities should not be considered as adverse because they occur indirectly.

A framework for interpreting data collected in tests must include five categories of endpoints: *a)* structural or neuropathological, *b)* neurophysiological, *c)* neurochemical, *d)* behavioural, and *e)* developmental neurotoxicity, all of them being possible indicators of neurotoxic effects.

[14] Based on information reviewed from IPCS/WHO (2001), Moser (2011), Nielsen et al., (2008); US EPA (1998)

1.1. Structural indicators of neurotoxicity

Structural indicators are defined as neuropathological lesions of the central or peripheral nervous system observed by microscopy, including immunohistochemistry and morphometry. Changes in brain weight are also considered as a biologically significant neurotoxic effect of the structural endpoint regardless of changes in body weight, because brain weight is generally assumed to be spared under conditions of mild undernutrition.

Various histological changes can result after exposure to neurotoxicants, for instance changes in nerve cell bodies (chromatolysis, vacuolisation and cell death), axons (swelling, degeneration and atrophy) and myelin sheath (folding, oedematous splitting and demyelination). The most distal processes of longer axons are especially vulnerable to certain neurotoxins resulting in a sort of dying-back axonopathy leading to a peripheral polyneuropathy, as occurs with some organophosphates (OPs). Dying neurons can undergo autophagocytosis or apoptosis with a condensation and dissolution of chromatin and transfer of chromatin into cytoplasmic autophagocytic vacuoles. Damage to the capillaries in the brain can lead to a swelling characteristic of encephalopathy. Gliotic activation is a neuroglial response that can be associated with sublethal insults to neurons. In relatively mild injury, the astrocytic properties supporting homeostasis can restore the damage. Injury sufficient to kill neurons is usually accompanied by a reactive change in astrocyte structure and function. Reactive astrocytes (astrogliosis) display hypertrophy, extend thick, long processes, elevated glutamine synthetase and oxido-reductive enzyme activity, and significantly increase their cytoplasmic content of glial fibrillary acidic protein (GFAP).

Although axonopathies and myelinopathies may correlate with decreased nerve conduction velocity (changes in motor or sensitive nerve latencies or peak amplitude; section 1.2.4), both endpoints are usually less sensitive than behaviour effects (section 1.4).

1.2. Neurophysiological indicators of neurotoxicity

Neurophysiological studies measure the electrical activity of the nervous system.

1.2.1. *Electroencephalography (EEG)*

There is a relationship between specific patterns of EEG waveforms and behavioural states ranging from alertness to sleep. Chemically-induced seizure activity detected in EEG is evidence of a neurotoxic effect. Sometimes, changes in the EEG pattern can precede alterations in other objective signs of neurotoxicity. However, EEG is of little help for the detection of subtle toxicant-induced dysfunction.

1.2.2. *Nerve conduction studies*

Nerve conduction studies are generally performed on peripheral nerves and are useful for investigating possible peripheral neuropathy. The most critical measurements are nerve conduction velocity, amplitude of electric potentials and refractory period. A decrease in amplitude of electric potential reflects a loss of active nerve fibres and may occur prior to a decrease in conduction velocity in the course of peripheral neuropathy. Hence, changes in amplitude of electric potential are better sensitive measurements of axonal degeneration. However, when the damage to nerve fibres is not extensive enough, the damage may not be reflected in these endpoints. Thus, the interpretation of data may be enhanced if evaluations such as nerve pathology or other structural measures are also included. A decrease in nerve conduction velocity may be indicative of demyelination. In cases where the primary toxic effect is axonal degeneration, nerve conduction velocity may not be reduced unless the fastest-conducting axons are affected. For this reason, a measurement of normal nerve conduction velocity does not necessarily rule out the presence of axonal degeneration.

1.2.3. *Electromyogram (EMG)*

EMG records and analyses the electric activity in skeletal muscles. Changes in the EMG include amplitude and firing frequency of spontaneous firing; evoked muscle responses to nerve stimulation

can be used to study alterations in a motor unit, which includes the alpha motor neuron, nerve root, peripheral nerve, neuromuscular junction and muscle. The single-fibre EMG (a more sensitive measure than the EMG with repetitive nerve stimulation) has been used to detect the blockage of neuromuscular transmissions induced by organophosphates (OPs).

1.2.4. *Sensory, motor, and other evoked potentials*

Evoked potentials reflect the function of the system under study, including sensory (visual, auditory or somatosensory), motor (involving motor nerves and innervated muscles) or other neural pathways in the central or peripheral nervous system. The latency (time from stimulus onset) and amplitude (voltage) of the positive and negative voltage peaks are commonly identified and measured. Increases in latencies can reflect deficits in nerve conduction, including demyelination or delayed synaptic transmission, and are indicators of a neurotoxic effect.

1.3. Neurochemical indicators of neurotoxicity

The integrity of nerve cells can be determined by using general biochemical indices, including endpoints of cellular toxicity, changes in energy-linked functions or changes in synthesis of cell constituents or proteins. Persistent or irreversible chemically-induced neurochemical changes are indicative of neurotoxicity. If these changes are expected to have neurophysiological, neuropathological or neurobehavioural correlates, then the neurochemical changes could be regarded as neurotoxic effects.

The assessment of cholinesterase-inhibiting chemicals should be done on a case-by-case basis using a weight-of-evidence approach in which all of the available data (e.g. brain, blood and other tissue cholinesterase activity, as well as the presence or absence of clinical signs) are considered in the evaluation. Plasma cholinesterase inhibition is considered not relevant.

The WHO-FAO Joint Meeting of Experts on Pesticide Residues (JMPR) has given recommendations on interpretation of cholinesterase inhibition. In line with the JMPR (1998), the Netherlands National Institute for Public Health and the Environment (RIVM) regards a statistically significant inhibition of AChE $\geq$ 20% in the central or peripheral nervous system and in erythrocytes as toxicologically relevant or 'adverse' (Luttik and Raaij, 2001). The inhibition of 20% may be considered with respect to the concurrent control group or with respect to the 'pre-exposure' values in the treated groups. The normal inter-individual variation for brain and erythrocyte AChE activity is roughly $\leq$ 20%. The US-EPA considers the value of 20% inhibition as a toxicological effect, but a 1997 policy document (Sette, 1997) states that only 'statistically significant decreases in brain AChE are generally considered toxicologically significant'. The document 'Guidelines for Neurotoxicity Risk Assessment' of the US-EPA (1998) does not include any statement on the toxicologically relevant level of AChE inhibition. Nevertheless, statistically significant inhibition of less than 20% or statistically insignificant inhibition above 20% indicates that a more detailed analysis of the data should be undertaken (Nielsen et al., 2008).

A subset of OPs also produces organophosphate-induced delayed neuropathy (OPIDN) after acute or repeated exposure. OPIDN is characterized by degeneration of long axons in both peripheral nerves and the spinal cord. Inhibition and aging of neuropathy target esterase (NTE) are associated with the initiation of OPIDN. However, OPIDN develops when more than 70% of NTE inhibition/aging has occurred (Lotti, 1991). This suggests that a certain degree of NTE inhibition, although not correlated with clinical neuropathy, can potentially cause OPIDN.

Chemically induced injury to the central nervous system can be accompanied by hypertrophy of astrocytes, leading to an increase in GFAP, the major intermediate filament protein in astrocytes. GFAP can either be measured biochemically in serum or by light microscopy with immunohistochemical stains (see Structural endpoints of neurotoxicity section). The interpretation of a

chemical-induced change in GFAP is greatly facilitated by corroborative data from the neuropathology evaluation.

1.4. Behavioural indicators of neurotoxicity

Behaviour reflects the integration of the various functional components of the nervous system, including sensory, motor and associative functions. Neurotoxic substances can adversely affect one or more of these functions or disrupt learning and memory processes, resulting in detrimental behavioural effects. Therefore, behaviour is generally considered a sensitive indicator of neuronal function, irrespective of the strong subjective bias that is implied in recording of behavioural effects, and may serve as a robust means of monitoring the neurotoxic potential of chemicals.

Changes in behaviour can arise from a direct effect of a toxicant on the nervous system or indirectly from its effects on other physiological systems. These changes may be observed in the absence of neuropathological evidence of structural damage. Tilson (1993) proposed two distinct tiers of functional testing of neurotoxicants. In the first tier, functional observational batteries (FOB) and motor activity tests may be used as a screening procedure to identify the neurotoxic effects of a chemical. The second tier involves many other measures of behaviour, including specialized tests of motor and sensory function and of learning and memory that allows for a more complete description of the effects and for dose-response relationships. Neurochemical and electrophysiological tests can also be included in the second tier based on the results of the core studies (Moser, 2011).

1.4.1. Functional Observational Battery (FOB).

A FOB is designed to detect and quantify major overt behavioural, physiological and neurological signs. It is based on the Irwin screen test, a gross neurological assessment of basic sensory-motor performance. For regulatory testing, the FOB provides information on effects at low doses and is sensitive to chemicals acting by different modes of action.

Sometimes, FOB data can be grouped into more than one neurological functions which do not necessarily map to specific regions of the nervous system. A typical FOB includes the following domains:

- *Motor function*. Motor function may be measured in terms of motor activity, coordination, equilibrium and strength. Motor effects have been classified into four types: weakness (decreased strength), incoordination, tremor and abnormal motor movements (myoclonia or spasms). Gait has been assessed as an index of coordination. Dose-dependent increases or decreases on the motor activity are common neurobehavioral endpoints reflecting pesticide neurotoxicity. Changes in motor activity often occur at exposure levels that affect other types of behaviour and at levels of exposure that do not produce gross signs of intoxication. However, interpretation of motor activity data in isolation can be problematic.
- *Sensory testing* used in first-tier screening involves either testing simple reflexes (e.g. pinna reflex) or evaluation of the motor response to a variety of sensory stimuli such as auditory, nociceptive or somatosensory (e.g. startle response, tail pinch response, splay reflex).
- *Autonomic function*: e.g. pupil response to light, pupil size (miosis, mydriasis), salivation, lacrimation, urination.

Many FOB tests are essentially clinical neurological examinations that rate the presence or absence of specific neurological signs. If neurological signs result only at the high dose and together with other overt signs of toxicity (e.g. systemic toxicity, large decreases in body weight, decreases in body temperature or debilitation) there is less persuasive evidence of a direct neurotoxic effect[15]. In contrast,

[15] The external scientific report (CFT/EFSA/PRAS/2012/07) concludes that great care is needed when considering clinical signs as a basis for CAG grouping.

when affected measures in a battery of tests are dose-dependent, the data are considered to be evidence of a neurotoxic effect, especially in the absence of systemic toxicity.

1.4.2. Specialized tests for neurotoxicity (that include specific neurobehavioral functions):

- *Motor Function*. Specialized tests used to assess muscle weakness include quantitative measures of limb grip strength, swimming endurance, suspension from a hanging rod, discriminative motor function and landing foot splay. Automated rotorod and quantitative gait assessments measure coordination, while rating scales and spectral analysis techniques are used to quantify tremor and other abnormal movements.
- *Sensory Function.* Gross perturbations of sensory function may not be sufficiently sensitive to detect subtle sensory changes. Several approaches have been devised to measure sensory deficits, including discriminated conditioning and startle reflex modification.
- *Cognitive Function*. Measurement of changes in learning and memory should be separated from other changes in behaviour that do not involve cognitive or associative processes (e.g. motor function, sensory capabilities, motivational factors). Examples of procedures to assess cognitive function include tests for spatial learning and memory, spatial or positional navigation of mazes, simple or complex conditioned responses and operant training of positively or negatively reinforced behaviours. Many of the current cognitive procedures are either water-based mazes or shock-motivated (i.e. simple conditioned avoidance of shock, such as passive avoidance). Many cognitive tests are conducted as part of developmental neurotoxicity assessment.

1.5. Developmental neurotoxicity (DNT)

The DNT study is a specialized type of developmental toxicity study designed to screen for adverse effects of pre- and postnatal exposure on the development and function of the nervous system and to provide dose–response characterizations of those outcomes. The US EPA (1998) developed test guidelines for assessing DNT of pesticides, among other environmental chemicals, as tier 2 studies for active substances showing any evidence of neurotoxicity, endocrine modulation or certain developmental or reproduction toxicity. Similarly, Regulation (EU) No 283/2013 of 1 March 2013 provides that, when indicated by observations in other studies or the mode of action of the test substance, supplementary studies or information may be required to provide information on the postnatal manifestation of effects such as developmental neurotoxicity. A reference to the OECD Test Guideline 426 is given in the Commission Communication 2013/C 95/01.
Both the prenatal and postnatal periods to the time of sexual maturation are critical developmental windows wherein functional competence may be disrupted as a result of pesticide exposure. This means that foetuses, infants and children may be differentially sensitive and not comparable between rodents and humans[16] because of the potential of these chemicals to impair the appearance or maturation of sensorimotor reflexes. While the developing nervous system is often more sensitive to insult (depending on the stage of development), the high rate of proliferation and regeneration in the developing nervous system may lead to greater recovery or plasticity (an ability of one portion of the nervous system to assume the function of an injured area), which could attenuate some injuries.

The behaviours measured in DNT studies include the development of motor, sensory, autonomic and cognitive (learning and memory) functions. Tests are performed at different time-points (weaning and adulthood) to cover all neurodevelopmental stages. Neuropathological and morphometric examinations are also performed to detect growth defects and morphologic abnormalities. Since many of the behaviours evaluated are the same as those used in adult rats, additional features in developmental studies include assessing the ontogeny of these behaviours or whether there are persistent behavioural changes lasting into adulthood. 'In vitro' DNT tests provide complementary

[16] The early postnatal stages of the development of nervous system in rodents correspond to the late prenatal phase in humans.

information since they are based on key events of brain development such as proliferation, differentiation, growth, synaptogenesis, myelination or apoptosis.

Functional deficits are observed at dose levels below those at which other indicators of developmental toxicity are evident or at minimally toxic doses in adults. Information available so far is inadequate to assume that developmental effects at doses causing minimal maternal toxicity result only from maternal toxicity; rather, it may be that the mother and developing organism are equally sensitive to that dose level.

2. Neurological disorders in humans potentially associated with long-term pesticides exposure

Acute (short-term) effects of overdoses of most pesticides are well characterised and they vary depending on the active substance, the dose and the susceptibility of the individual exposed. By contrast, the scientific community is less certain about the long-term health effects that may result from repeated exposures over time. Despite this, there is growing epidemiological data supporting an association between neurological effects and pesticide exposure. Recent studies point to some possible long-term health effects from repeated low-level exposure to pesticides, although this data are still inconclusive.

A recent meta-analysis suggests a relationship between pesticide exposure and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. Thus, exposure to herbicides and insecticides could increase the risk of Parkinson's disease (van der Mark et al., 2012). However, a prospective study has found a weak but dose-related association between pesticide exposure and risk for Parkinson's disease, although it was based on a small number of cases (Kenborg et al., 2012). It is known from animal experiments that rotenone can cause symptoms that are typical for Parkinson's disease. These symptoms have pathological correlates that include, for example, cytoplasmic inclusions in the neurons of the *substantia nigra*, a region of the midbrain involved in the pathogenesis of Parkinson's disease. Rotenone can lead to a progressive degeneration and loss of nigrostriatal dopaminergic neurons (BfR, 2006). The mechanisms underlying rotenone-induced Parkinson's symptoms in mice (Pan-Montojo et al., 2012) can therefore be interpreted as further indications of a possible association between rotenone exposure and Parkinson's-like disease. Other meta-analysis has found that exposure to pesticides as a group is associated with the development of amyotrophic lateral sclerosis, although the analysis of Agricultural Health Study (AHS) data points in particular to organochlorine pesticides use (Kamel et al., 2012; Malek et al., 2012).

Occupational pesticide exposure has been reported to increase the risk of dementia and Alzheimer disease in late life (Hayden et al., 2010). Environmental pesticide exposure has also been linked to the development of Alzheimer's disease (Parrón et al., 2011).

There is growing evidence that the nervous system in young animals is particularly vulnerable to some insecticides, hence early life environmental exposure to pesticides could play a critical role in the onset of age-related diseases. There is some experimental basis for this because postnatal exposure to cypermethrin in rats enhances the susceptibility of animals to dopaminergic neurodegeneration if rechallenged during adulthood (Singh et al., 2012).

Human epidemiological studies have found associations between environmental exposure to pesticides and prenatal and postnatal adverse effects. The results reported by a small number of longitudinal birth cohort studies suggest that prenatal exposure to OP pesticides in the early stages may adversely affect the developing nervous system of the child, in particular the cognitive function (e.g. pervasive developmental disorders, delays in cognitive development and attention deficit). However, the experimental evidence for adverse neurodevelopmental effects in children after postnatal exposure to these compounds is less persuasive. Behavioural problems, poorer short-term memory and motor skills, and longer reaction time have been observed in cross-sectional studies (London et al, 2012).

3. Establishment of CAG for toxic effects on the nervous system

3.1. CAG level 1

In the DTU report (2012), CAG level 1 was defined as 'Toxicity to the nervous system' as the nervous system was identified as the relevant organ and target tissue.

3.2. CAG level 2

The DTU proposed three distinct CAG 2 levels:

- Functional changes related to the motor division (53 pesticides), involving effects on the movement of muscles (motor activity, ataxia, choreoathetosis, abnormal gait, paralysis, neuromuscular dysfunction), effects on locomotion (decreased locomotion) and neuropathy (peripheral neuropathy, particularly OP-induced delayed polyneuropathy).
- Effects on reflex action (16 pesticides), which involves either sensory (afferent stimuli) or motor (efferent response) division of the nervous system in an involuntary manner.
- Effect on cognition (7 pesticides), an intellectual process that involves all aspects of perception, thinking, reasoning and remembering.

The DTU did not consider the presence of vacuoles in brain, changes in relative brain weight and induction of neoplasm (particularly astrocytomas) as relevant for CAG level 2 as these three effects were viewed as non-adverse, not to be treatment-related and not to be statistically significantly different from control groups, respectively. Besides, brain neoplasms (astrocytomas) are rarely described in experimental studies and no statistically significant differences have been found between treated and control groups, hence they should not be taken into account for CRA.

The active substances identified as neurotoxic by the RIVM contractor (ANSES/ICPS/RIVM, 2013) have been included in a reporting table along with relevant information on critical endpoints, NOEL/LOEL in the most sensitive species and the neurotoxic mode of action. However, for 32 pesticides identified as neurotoxic the specific mode of action is still unknown. RIVM proposed the following effects (indicators) for grouping:

- AChE inhibition.
- Tremor.
- Salivation.
- Ataxia (abnormal gait, among other synonyms).
- Motor activity (impaired mobility, open field activity, ambulatory activity, hypo/hyperactivity, decreased rearing activity).
- Reflex/sensory response (hypersensitivity, absence of pupil response, lack of touch response, lack of startle response, negative air drop, decreased analgesic reflex).
- Cognition (learning, memory, maze test performance, performance in active/passive avoidance test).

A number of effects (such as prostration, convulsion, opisthotonus, laboured breathing, tachypnea, hunched position/posture, dyspnoea, exopthalmos, lethargy, piloerection, curved body position, coma, hypothermia, vomiting and alopecia) were considered as non-specific by RIVM, often secondary to general systemic toxicity after high doses rather than a neurotoxic property of the pesticide. Accordingly, they are not deemed appropriate for establishing CAGs unless they appeared at low doses. Piloerection can also be considered an autonomic sign.

The evaluation summarised in the table for hazard identification and characterisation (see chapter 7) combines information from the DTU report (DTU, 2012) and RIVM (ANSES/ICPS/RIVM, 2013). The information was also combined with information hazard identification and characterisation from

US-EPA and IPCS/WHO reports. The following toxicological effects are therefore recommended for the nervous system CAG 2:

CAG 2.1. Functional effects on motor division
CAG 2.2. Functional effects on sensory division (including sensory-motor reactivity or reflex action)
CAG 2.3. Functional effects on autonomic division
CAG 2.4. Neurophysiological alterations
CAG 2.5. Neurochemical endpoints (AChE inhibition)
CAG 2.6. Neuropathological endpoints

This approach does not meet the systematic order described in section 1 of this Annex because the available toxicological data failed to contain information for some of the categories of endpoints described, as occurred for cognitive (learning and memory) and developmental neurotoxicity indicators of adverse effects. In addition, data related to neurochemical endpoints are limited to acetylcholinesterase inhibition in blood or brain and only few neurophysiological and histopathological data are available. Since most of experimental data were collected from FOB studies, behavioural data (motor, sensory and autonomic functions) predominate.

Further refinement of the above mentioned adverse effects was made for the hazard characterisation (step 2).

3.3. CAG level 3. Data collection

The majority of the data collection for neurotoxicity had been performed by DTU and later re-evaluated by RIVM prior to the finalization of step 1 and 2. A minor part of the data collection was performed by EFSA at a later stage. This would explain any differences in the approach taken for the data collection between DTU, RIVM and EFSA.

From the 224 active substances included in Annex I of the Council Directive 91/414/EEC prior to 31st May 2009, only 67 were identified by DTU as having effects on the nervous system. Then, from 31st May 2009 until 1st of January 2012, 60 new active substances were added to Annex I.

In total, 130 active substances have been scrutinised by RIVM for their neurotoxic potential, including the 3 substances not previously assessed by DTU (flurtamone, oxadiargyl and pyridate), although only pyridate showed potentially neurotoxic effects. Evaluation of the 60 pesticides added to Annex I during the period 31st of May 2009 until 1st of January 2012 revealed that 9 had clear neurotoxic properties. However, two of these (zinc phosphide and aluminium sulphate) were not included in the final reporting table as their targeted use (rodenticide and bactericide on cut flowers, respectively) does not involve edible plants and it is unlikely to find significant residues in plant or animal products. In fact, no MRLs have been set for these substances in either plant or animal products.

After performing a re-evaluation of all data, RIVM found no convincing evidence for 8 pesticides identified as neurotoxic by the DTU report (dimoxystrobin, dinocap, florasulam, fosetyl-aluminium, phenmedipham, prosulfocarb, sulcotrione and triflusulfuron-methyl). Although some of them are able to inhibit cholinesterase or to develop some neurotoxic effects, they are of slight magnitude, non-specific or secondary to high-dose systemic toxicity. Accordingly, they were not considered for grouping purposes. The RIVM database included the active compounds desmedipham and chlorpropham, however given that they are structurally very similar to each other and to phenmedipham (all of them are phenylcarbamate herbicides), their toxicological profile was scrutinised and no evidence of neurotoxic potential was found. Nevertheless, desmedipham and chlorpropham remain in the grouping table because their DARs have identified several specific endpoints of neurotoxicity.

A number of active substances excluded by the DTU report showed neurotoxic effects just as a consequence of a generalised secondary effect or at dose levels close to those that produced mortality. This is the case of aluminium phosphide, benzoic acid, benthiavalicarb, bifenazate, copper compounds, cyromazine, difenoconazol, etofenprox, etoxazole, fenpyroximate, magnesium phosphide, metamitron, metribuzin, pymetrozine, pyriproxyfen, tebufenpyrad, thiophanate-methyl and tolylfluanid. Accordingly, none of the compounds showing neurotoxic effects as a result of general toxicity were included in the grouping table. By contrast, other active substances also excluded by DTU, such as tebuconazole and tetraconazole, were identified as producing some toxic effects on the nervous system. Thus, both of them were considered for grouping.
In summary, a total of 68 active substances placed on Annex I of the Council Directive 91/414/EEC prior to 1st of January 2012 were identified as having neurotoxic properties by RIVM.

3.4. CAG level 4. Grouping

Different approaches can be used for grouping active substances, like structural similarities of the parent compounds (or their toxic metabolites), degree of knowledge of their mechanism of toxic action or a common toxic effect. The approaches might be different from each other if used for risk assessment or for MRL setting. Despite the fact that the most appropriate criteria for further refining cumulative assessment groups are based on the mode of action, this information is unknown for many of the active substances identified as neurotoxic. Although a number of neurotoxic pesticides share common specific effects, the underlying toxic mechanism at biochemical level is often unknown.

For this reason, grouping of active substances was based on observed specific indicators for neurotoxic effects, which is an imperfect criterion since they can differ as a function of the dose or the duration of the treatment. Accordingly, this preliminary approach must be considered as non-definitive grouping that needs further refinement, when knowledge gaps related to the mode of action are filled. In addition, this would represent the most precautionaryapproach that takes advantage of the available toxicological database on neurotoxic effects of many active substances. One important limitation of this approach is the lack of a clear definition of a common endpoint in terms of identical cell types affected, identical pathology or identical biological response in the same target organ.

The testing methodology for DNT has not been a requirement in the European Union and this circumstance contributes to the lack of characterization of these specific effects. Specific DNT testing has not been conducted according to OECD guideline 426 since it was developed in 2007. Accordingly, the exercise of DNT grouping cannot be considered fully correct because the experimental studies available for risk assessment were restricted to developmental and reproductive toxicity testing. For this reason, DNT effects have not been grouped together with the rest of the indicators of neurotoxic effects.

Two tables have been developed for grouping active substances, one for acute and the other for chronic indicators of neurotoxic effects. The latter also includes data from subchronic studies. Data were tabulated according to the level of organisation of the nervous system (category of neurotoxic endpoints), the specific indicator of toxic effect, the active substance, its mode/mechanism of action and the lowest experimental toxicity indices (NOAEL and LOAEL) for each specific indicator of neurotoxicity. Sometimes the indices derived from several toxicological studies reported in the DARs

According to the criteria employed for grouping, each active substance may show more than one NOAEL/LOAEL, depending on the number of toxic effects identified in the databases used. A number of pesticides may share the same toxic endpoints via similar or dissimilar mechanism of action and therefore have been grouped together; however for many of them the mechanisms of action are unknown. Certain neurotoxic signs (such as tremors, lack of motor coordination, ataxia, decreased motor activity and abnormal posture) may result from the activation of the nicotinic receptor. Thus, these signs can appear after exposure to pesticides showing different mechanisms of toxic action but a similar mode of action (modulation of cholinergic synaptic transmission).

The following considerations have been taken into account for grouping active substances:

- Given the complexity of the Nervous system, most DARs rely on a functional observational battery (FOB) as indicators of specific effects.
- Indicators for specific neurotoxic effects selected for the grouping table are based on the information collected and included in the RIVM database and in an additional database developed by EFSA containing compounds excluded by the DTU report. Since no one cognitive indicator of effects has been identified in the databases, this neurologic domain has not been considered separately.
- Only studies where the active substance was administered by oral route have been considered for grouping.
- Data from reproductive toxicity studies were not considered since toxicological endpoints were not always clearly reported as pertaining to dams or pups.
- Developmental toxicity studies were also disregarded as they did not properly assess toxic effects on the developmental nervous system.
- Sometimes, the same specific effect is reported with different names, depending on the DARs and the experimental studies. This has been also acknowledged by the DTU and RIVM.
- Some effects were described in a very general way (e.g. head shaking, head movements) and they have been reinterpreted (e.g. choreoathetosis).
- Non-specific or secondary effects are often described in the database but they have not been considered for grouping. This is the case of emesis, hypothermia, headache, exopthalmus, aggression, opisthotonus, nystagmus and vocalisation. The same applies to those effects that occur after administration of high doses of the active substances resulting in general toxicity, such as laboured breathing, emaciation or prostration.
- Some effects (e.g. convulsion) may occur after high doses, as a result of general toxicity. However, they can be also a specific effect of an active substance (e.g. tonic-clonic convulsions of pyrethroids).
- Some NOAEL for acute effects do not derive from acute neurotoxicity studies but they are based on 14-, 28- or 90-day studies, with observations being performed on the first day of dosing.
- Salivation has been considered as an autonomic effect. Nonetheless, it is also a particular effect produced by type II (α-cyano) pyrethroids, the so-called CS (choreoatetosis-salivation) syndrome.
- Abnormal posture of animals, especially lateral recumbency, has been considered as a neurotoxic effect since it may be observed in cerebellar lesions.
- Decreased limb strength may be associated with dragging of the feet along the floor, hindlimb paresis/paralysis, foot splay, hopping gait and eventually ataxia. All these clinical outcomes are in fact a continuum of effects that can or cannot be considered as a single effect for the purpose of grouping.
- Several experiments have been carried out with a given pesticide and different animal species and dose regimes. The lowest NOAEL and LOAEL were selected for each specific indicator of neurotoxicity based on combined data from two or more studies. Thus, some NOAELs and LOAELs derive from different studies or reports.
- Studies considered unacceptable by EFSA have not been taken into consideration (e.g. not guidelines, not GLP).
- Modes of action are based primarily on the information included in RIVM database and for some active substances the open scientific literature has been reviewed.
- The neurochemical endpoints represent a level of grouping for neurotoxic substances based on mechanism of action. However, to keep consistency in the group methodology, the cumulative assessment grouping was limited to phenomenological observations following the motor, sensory and autonomic division of the nervous system. Indeed, the AChE inhibitors play a prominent role in the risk assessment and grouping neurochemical endpoints would result in an increased sensitivity for some substances and this grouping should be used for further refinement where this mechanism of action is recognised.

- Molinate, desmedipham and toclofos-methyl were not considered for grouping within the 'neurochemical endpoints' because they are atypical cholinesterase inhibitors showing a weak AChE inhibition potential. For tolclofos-methyl, the only neurotoxicity data collected by RIVM was AChE inhibition. Although this compound is an organothiophosphate ester used as herbicide, there is a lack of consistency between older and new studies regarding AChE inhibition. Besides, clinical signs of cholinergic overstimulation were not observed in experimental studies. On the other hand, it has been acknowledged the unlikelihood of being exposed to tolclofos-methyl residues through the diet (EPA, 2013)

- Neuropathological endpoints were considered relevant only for chronic cumulative assessment grouping. This is because, for chronic treatments, some active substances were only showing neuropathological findings as well as for some other active substances, neuropathological endpoints were the most sensitive.

B. DATA COLLECTION FOR EFFECTS ON THE NERVOUS SYSTEM

B.1. Data collection for neurotoxicity - RIVM

See separate file.

B.2. Data collection for neurotoxity – EFSA

See separate file.

C. ACUTE CAGS FOR THE NERVOUS SYSTEM

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
Functional effects on Motor division	Ataxia	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Deltamethrin	1	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Formetanate	1	10	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Fenamiphos	1.25	2.31	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Abamectin	1.5	6	Presumed	Binding to GABA receptors and GABA gated ion channels.
	Ataxia	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Ataxia	Alpha-Cypermethrin	2.3	6.8	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Tefluthrin	5	10	Presumed	Type I pyrethroid. Opening of sodium channels in the nervous system
	Ataxia	Fosthiazate	5.4	26.8	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Cypermethrin	7.5	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Phosmet	9	36	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Milbemectin[1]	10	30	Presumed	Binding to GABA receptors and GABA gated ion channels
	Ataxia	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Ataxia	2,4-D	< 15	15	Unknown	Cell and mitochondrial membrane damage, uncoupling of oxidative phosphorylation and interference of cellular metabolism?
	Ataxia	Ziram	< 15	15	Presumed	Neurotoxic effect might be due to its metabolite CS_2
	Ataxia	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Indoxacarb	50	100	Presumed	Presumed blocking of sodium channels (in insects)
	Ataxia	Clothianidin	60	177	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Ataxia	Flufenacet	< 75	75	Presumed	Metabolic lesions due to limitations in glutathione interdependent pathways and antioxidant stress
	Ataxia	Tebuconazole[1]	< 100	100	Unknown	Increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways?
	Ataxia	Dicamba	< 300	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
	Ataxia (poor coordination)	Tetraconazole[1]	< 300	300	Unknown	Neurotoxic potential of 1,2,4-triazole, a metabolite common to a number of triazole-derivative pesticides
	Choreoatetosis	Cyfluthrin	1	2.5	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Choreoatetosis	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Convulsions	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Convulsions	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Convulsions	Clothianidin	60	177	Known	Nicotinic acetylcholine receptor (nAChR)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
						agonist
	Convulsions	Pirimiphos-methyl	150	1500	Known	Inhibition of acetylcholinesterase (AChE)
	Higher grip strength	Thiamethoxam[1]	100	500	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Hunched posture	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Hunched posture	Pirimicarb	10	40	Known	Inhibition of acetylcholinesterase (AChE)
	Hunched posture	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Hunched posture	Ziram	< 15	15	Presumed	Neurotoxic effect might be due to its metabolite CS_2
	Hunched posture	Indoxacarb	50	100	Presumed	Presumed blocking of sodium channels (in insects)
	Hunched posture	Glufosinate	100	500	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Hunched posture	Tetraconazole[1]	< 300	300	Unknown	Neurotoxic potential of 1,2,4-triazole, a metabolite common to a number of triazole-derivative pesticides
	Increased motor activity	Triadimenol (a metabolite of Triadimefon)	2	35	Unknown	Increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways?
	Landing-foot splay	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Landing-foot splay	Fipronil	0.5	5	Presumed	Blocking the passage of chloride ions through the GABA receptor
	Landing-foot splay	Deltamethrin	1	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Landing-foot splay	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Landing-foot splay	Tefluthrin	5	10	Presumed	Type I pyrethroid. Opening of sodium channels in the nervous system
	Landing-foot splay	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Landing-foot splay	Indoxacarb	50	100	Presumed	Presumed blocking of sodium channels (in insects)
	Muscle fasciculation	Methiocarb	<2.5	2.5	Known	Inhibition of acetylcholinesterase (AChE)
	Motility disturbances	Tebuconazole[1]	< 100	100	Unknown	Increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways?
	Recumbency	Tebuconazole[1]	< 100	100	Unknown	Increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways?
	Recumbency	Tetraconazole[1]	< 300	300	Unknown	Neurotoxic potential of 1,2,4-triazole, a metabolite common to a number of triazole-derivative pesticides
	Reduced grip strength	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Reduced grip strength	Thiram	5	150	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Reduced grip strength	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Reduced grip strength	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced grip strength	Indoxacarb	50	100	Presumed	Presumed blocking of sodium channels (in insects)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Reduced grip strength	Dicamba	< 300	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
	Reduced motor activity	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced motor activity	Beta-Cyfluthrin	0.5	2	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Reduced motor activity	Lambda-Cyhalothrin	0.52	1.3	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Reduced motor activity	Formetanate	1	10	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced motor activity	Thiacloprid	3.1	11	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Reduced motor activity	Ethoprophos	5	10	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced motor activity	Acetamiprid	10	30	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Reduced motor activity	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced motor activity	Pyridate	20	60	Unknown	
	Reduced motor activity	Tri-allate	36	72	Unknown	
	Reduced motor activity	Chlorpropham	50	125	Unknown	Impairment of mitochondrial function related to oxidative phosphorylation leading to ATP depletion?
	Reduced motor activity	Indoxacarb	50	100	Presumed	Presumed blocking of sodium channels (in insects)
	Reduced motor activity	Mepiquat	58	174	Presumed	Activation of nicotinic and muscarinic acetylcholine receptors
	Reduced motor activity	Clothianidin	60	177	Known	Nicotinic acetylcholine receptor (nAChR) agonist

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Reduced motor activity	Flufenacet	< 75	75	Presumed	Metabolic lesions due to limitations in glutathione interdependent pathways and antioxidant stress
	Reduced motor activity	Dicamba	< 300	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
	Tremor	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Fluquinconazole	0.45	1.79	Unknown	
	Tremor	Methomyl	0.75	2	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Deltamethrin	1	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Formetanate	1	10	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Tremor	Methiocarb	< 2.5	2.5	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Tefluthrin	5	10	Presumed	Type I pyrethroid. Opening of sodium channels in the nervous system
	Tremor	Phosmet	9	36	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Acetamiprid	10	30	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Tremor	Milbemectin[1]	10	30	Presumed	Binding to GABA receptors and GABA gated ion channels
	Tremor	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Pyrethrins	20	63	Known	Opening of sodium channels in the nervous system

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Tremor	Imidacloprid	23.5	45.4	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Tremor	Metaldehyde	< 75	75	Unknown	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?
Functional effects on Sensory division	Abnormal righting reflex. Increased tail flick latency time	Dicamba	< 300	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
	Absence of pupil response	Dimethoate	2	20	Known	Inhibition of acetylcholinesterase (AChE)
	Approach response, tail pinch response, air righting reflex.	Fipronil	5	25	Presumed	Blocking the passage of chloride ions through the GABA receptor
	Decrease in acoustic startle response amplitude	Deltamethrin	< 1	1	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Decreased arousal	Clothianidin	< 100	100	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Decreased touch responses, tail pinch response and impaired righting.	Beta-Cyfluthrin	2	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Diminished reaction to tail pinch test, abnormal response to visual placing	Formetanate	1	10	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	test, auditory startle response					
	Exaggerated startle response	Pyrethrins	63	200	Known	Opening of sodium channels in the nervous system
	Handling reactivity, approach response, startle response, air righting	Thiram	5	150	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Hypersensitivity to noise	Cypermethrin	7.5	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Increased reaction to touch, increased reaction to tail pinch	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Increased reactivity	Imidacloprid	42	151	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Lack of pupillary reflex	Mepiquat	174	697	Presumed	Activation of nicotinic and muscarinic acetylcholine receptors
	No reaction to tail-pinch stimulus	Methomyl	1	1.9	Known	Inhibition of acetylcholinesterase (AChE)
	Poor reflexes	Tebuconazole[1]	< 100	100	Unknown	
	Reduced splay reflex	Abamectin	0.5	1.5	Presumed	Binding to GABA receptors and GABA gated ion channels.
	Reduced righting reflex, reduced toe/tail	Metaldehyde	150	250	Unknown	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	pinch response					
	Righting reflex	zeta-Cypermethrin	10	50	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Righting reflex, tail pinch	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Uncoordinated landing in the righting reflex	Thiamethoxam[1]	100	500	Known	Nicotinic acetylcholine receptor (nAChR) agonist
Functional effects on autonomic division	Lacrimation	Methomyl	0.75	2	Known	Inhibition of acetylcholinesterase (AChE)
	Lacrimation	Methiocarb	< 2.5	2.5	Known	Inhibition of acetylcholinesterase (AChE)
	Lacrimation	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Lacrimation	Tri-allate	< 500	500	Unknown	
	Miosis	Formetanate	1	10	Known	Inhibition of acetylcholinesterase (AChE)
	Miosis	Fenamiphos	1.52	2.31	Known	Inhibition of acetylcholinesterase (AChE)
	Miosis	Fipronil	5	50	Presumed	Blocking the passage of chloride ions through the GABA receptor
	Miosis	Pirimicarb	10	40	Known	Inhibition of acetylcholinesterase (AChE)
	Miosis	Ethephon	<500	500	Known	Inhibition of acetylcholinesterase (AChE)
	Mydriasis	Deltamethrin	1	2.5	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Mydriasis	Thiacloprid	53	109	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Mydriasis	Metaldehyde	< 75	75	Unknown	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?
	Piloerection	Fenamiphos	1.52	2.31	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Salivation	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Methomyl	0.75	2	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Esfenvalerate	1.8	1.9	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Salivation	Beta-Cyfluthrin	2	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Salivation	Cyfluthrin	2.5	7.5	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Salivation	Methiocarb	< 2.5	2.5	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation (accompanied by vomiting)	Milbemectin[1]	3	10	Presumed	Binding to GABA receptors and GABA gated ion channels
	Salivation	Alpha-Cypermethrin	4	10	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Salivation	Phosmet	9	36	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Ethoprophos	12	25	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Dimethoate	20	200	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Pyridate	30	80	Unknown	
	Salivation	Pyrethrins	63	200	Known	Opening of sodium channels in the nervous system
	Salivation (accompanied by vomiting and retching)	Chlorpropham	125	625	Unknown	Impairment of mitochondrial function related to oxidative phosphorylation leading to ATP depletion?
	Salivation	Tebuconazole[1]	250	500	Unknown	
	Salivation	Tri-allate	< 500	500	Unknown	

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	Urination	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	Urination	Thiram	5	150	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Urination	Acetamiprid	10	30	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Urination	Cypermethrin	20	60	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Urination	Indoxacarb	50	100	Presumed	Presumed blocking of sodium channels (in insects)
	Urination	Thiacloprid	53	109	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Urination	Pyrethrins	63	200	Known	Opening of sodium channels in the nervous system
	Urination	Flufenacet	< 75	75	Presumed	Metabolic lesions due to limitations in glutathione interdependent pathways and antioxidant stress
Neurochemical end-points	AChE inhibition (brain)	Formetanate	0.1	1	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Methomyl	0.25	0.5	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Fosthiazate	0.5	5.4	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Pirimicarb	< 2	2	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Fenamiphos	2.7	9.3	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Phosmet	4.5	22.5	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition	Pirimiphos-methyl	15	150	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw	LO(A)EL mg/kg bw	Mode/mechanism of action	
	(brain)					
	AChE inhibition (erythrocytes)	Formetanate	0.1	1	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Oxamyl	0.1	0.75	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Methomyl	0.25	0.5	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Methiocarb	< 0.5	0.5	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Dimethoate	1	2	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Ethoprophos	< 5	5	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Malathion	10	> 10	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Pirimiphos-methyl	15	150	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Ethephon	22	66	Known	Inhibition of acetylcholinesterase (AChE)

NO(A)EL/LO(A)EL are expressed in mg/kg/day or mg/kg
[1]Substance excluded by DTU

D. CHRONIC CAGS FOR THE NERVOUS SYSTEM

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
Functional effects on motor division	Ataxia	Abamectin	0.25	0.5	Presumed	Binding to GABA receptors and GABA gated ion channels
	Ataxia	Fluquinconazole	0.44	4.77	Unknown	
	Ataxia	Lambda-Cyhalothrin	0.5	3.5	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Fosthiazate	0.54	5.4	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Deltamethrin	1	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Oxasulfuron	1.3	11	Unknown	
	Ataxia	Oxamyl	1.69	15.3	Known	Inhibition of acetylcholinesterase (AChE)
	Ataxia	Molinate	1.8	13	Unknown	Inhibition of aldehyde dehydrogenase and further accumulation of endogenous neurotoxic metabolites of neurotransmitters (e.g. dopamine)
	Ataxia	Beta-Cyfluthrin	2	8.9	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Alpha-Cypermethrin	2.3	6.8	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Cyfluthrin	2.4	11	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia	Indoxacarb	2.6	14	Presumed	Presumed blocking of sodium channels (in insects)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Ataxia	Cypermethrin	3.7	15	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Ataxia (Hind-limb wheelbarrowing)	Propineb	4.3	41.4	Unknown	Actin depolymerisation, disruption of cytoskeletal organisation and further acetylcholine release.
	Ataxia	Glufosinate	4.5	8.4	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Ataxia	Fenpropidin	5	20	Unknown	Disturbance of cholesterol supply/synthesis by Schwann cells
	Ataxia	Desmedipham	< 9.6	9.6	Unknown	
	Ataxia	Chlormequat	10	32	Unknown	Weak agonistic activity on muscarinic and nicotinic receptors
	Ataxia	Carbetamide	30	150	Unknown	
	Ataxia	Pyrethrins	30	86	Known	Delaying of the closing of sodium channels in the nervous system
	Ataxia	Tri-allate	36	72	Unknown	
	Choreoatetosis (Ruffling of body, pawing)	tau-Fluvalinate	0.5	1	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Choreoatetosis (Repetitive pawing)	Beta-Cyfluthrin	2	8.9	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Convulsions	Fipronil	0.019	0.057	Presumed	Blocking the passage of chloride ions through the GABA receptor
	Convulsions	Lambda-Cyhalothrin	0.5	3.5	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Convulsions	Lufenuron	1.9	20	Unknown	

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Convulsions	Glufosinate	4.5	8.4	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Convulsions	Mepiquat	32	95	Presumed	Activation of nicotinic and muscarinic acetylcholine receptors
	Deficits in stride width	Flufenacet	1.14	27	Presumed	Metabolic lesions due to limitations in glutathione interdependent pathways and antioxidant stress
	Dragging of hind feet and tail	Thiram	5.3	20	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Hunched posture	Fluquinconazole	0.44	4.77	Unknown	
	Hunched posture	Oxamyl	1.69	15.3	Known	Inhibition of acetylcholinesterase (AChE)
	Hunched posture	Lambda-Cyhalothrin (study performed with Cyhalothrin)	1.8	9.2	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Hunched posture	Indoxacarb	2.6	14	Presumed	Presumed blocking of sodium channels (in insects)
	Hunched posture	Acetamiprid	7.1	17.5	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Hunched posture	Pirimiphos-methyl	9	36	Known	Inhibition of acetylcholinesterase (AChE)
	Hunched posture	Tetraconazole[1]	17	65	Unknown	Neurotoxic potential of 1,2,4-triazole, a metabolite common to a number of triazole-derivative pesticides
	Hyperactivity	Lambda-Cyhalothrin (study performed with Cyhalothrin)	1.8	9.2	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Hyperactivity	Spinosad	67.5	185	Unknown	

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Hyperactivity followed by hypoactivty	Glufosinate	4.5	8.4	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Increased motor activity	Beta-Cyfluthrin	2	8.9	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Increased motor activity	Triadimenol (a metabolite of Triadimefon)	3.4	45	Unknown	Increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways?
	Landing-foot splay	Fenpropimorph	0.8	8.5	Unknown	
	Landing-foot splay	Deltamethrin	1	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Landing-foot splay	zeta-Cypermethrin	5	26	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Landing-foot splay	Tri-allate	32	177	Unknown	
	Lateral position	Mepiquat	32	95	Presumed	Activation of nicotinic and muscarinic acetylcholine receptors
	Limited use of hindlilmbs	Isoxaflutole	20	300	Unknown	
	Muscle weakness	Chlorpyrifos-methyl	10	50	Known	Inhibition of acetylcholinesterase (AChE)
	Muscle weakness	Methiocarb	2.2	8.6	Known	Inhibition of acetylcholinesterase (AChE)
	Paralysis	Thiram	5.3	20	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Paralysis	Metaldehyde	< 10	10	Presumed	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Paralysis	Mancozeb	49	328	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Paresis limbs	Fenpropidin	5	20	Unknown	Disturbance of cholesterol supply/synthesis by Schwann cells
	Paresis limbs	Pyrethrins	30	86	Known	Delaying of the closing of sodium channels in the nervous system
	Paresis limbs	Maneb	75	200	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Ptosis	Oxamyl	1.69	15.3	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced grip strength	Metiram	25.4	81.4	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Reduced grip strength	Beta-Cyfluthrin	2	8.9	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Reduced grip strength	Ethoprophos	2.65	27.11	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced grip strength	Esfenvalerate	3.2	7.3	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Reduced grip strength	Tri-allate	32	177	Unknown	
	Reduced motor activity	Ethoprophos	2.65	27.11	Known	Inhibition of acetylcholinesterase (AChE)
	Reduced motor activity	Fenpropidin	5	20	Unknown	Disturbance of cholesterol supply/synthesis by Schwann cells
	Reduced motor activity	zeta-Cypermethrin	5	26	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Reduced motor activity	Desmedipham	< 9.6	9.6	Unknown	
	Reduced motor	Clothianidin	35.8	52.3	Known	Nicotinic acetylcholine receptor (nAChR)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	activity					agonist
	Reduced motor activity	Dicamba	50	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
	Reduced motor activity	Tebuconazole[1]	100	300	Unkown	
	Reduced motor activity	Milbemectin[1]	101	213		Binding to GABA receptors and GABA gated ion channels
	Transient hyperactivity followed by hypoactivty	tau-Fluvalinate	0.5	1	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Abamectin	0.25	0.5	Presumed	Binding to GABA receptors and GABA gated ion channels
	Tremor	Fluquinconazole	0.44	4.77	Unknown	
	Tremor	Lambda-Cyhalothrin	0.5	3.5	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Tefluthrin	0.5	1.5	Presumed	Type I pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Fenamiphos	0.56	1.7	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Deltamethrin	1	10	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Formetanate	1	3	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Methiocarb	2.2	8.6	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Alpha-Cypermethrin	2.3	6.8	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Ethoprophos	2.65	27.11	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Pirimicarb	3.5	10	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Tremor	Cypermethrin	3.7	15	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Tremor	Glufosinate	4.5	8.4	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Tremor	Methomyl	6	16	Known	Inhibition of acetylcholinesterase (AChE)
	Tremor	Desmedipham	< 9.6	9.6	Unknown	
	Tremor	Imidacloprid	23.5	45.4	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Tremor	Pyrethrins	30	86	Known	Delaying of the closing of sodium channels in the nervous system
	Tremor	Tri-allate	36	72	Unknown	
	Tremor	Dicamba	50	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
Functional effects on sensory division	Decrease in alertness and/or startle response	Glufosinate	< 521	521	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Decreased pupil reactivity	Abamectin	0.25	0.5	Presumed	Binding to GABA receptors and GABA gated ion channels
	Decreased responsiveness to sensory stimuli, increase in click response	tau-Fluvalinate	2	6	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Diminished reflex response	Chlormequat	50	62.5	Unknown	Weak agonistic activity on muscarinic and nicotinic receptors
	Hindlimb flexor reflex	Oxasulfuron	83	199	Unknown	
	Hyperreactivity, absent pupillary	Oxamyl	1.69	15.3	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	response					
	Hyperreactivity	Indoxacarb	2.6	14	Presumed	Presumed blocking of sodium channels (in insects)
	Hyperreactivity	Cymoxanil	30	90	Unknown	
	Hyperreflexic patellar reflexes	Molinate	10	50	Unknown	Inhibition of aldehyde dehydrogenase and further accumulation of endogenous neurotoxic metabolites of neurotransmitters (e.g. dopamine)
	Hypersensitivity to noise	Deltamethrin	4	14	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Hypersensitivity to noise	Cypermethrin	5	15	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Hypersensitivity to noise	Esfenvalerate	15	25	Presumed	Appear to exhibit characteristics of both type I and type II pyrethroids. Interaction with voltage-gated sodium channels in the nervous system
	Hypo-reactivity, reduced reaction to movement and sound, hyperreactivity	Flufenacet	27	59	Presumed	Metabolic lesions due to limitations in glutathione interdependent pathways and antioxidant stress
	Increased alertness, impaired righting reflex	Tri-allate	32	177	Unknown	
	Increased reactivity, exaggerated auditory response	Beta-Cyfluthrin	2	8.9	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Increased response to sound	Tefluthrin	1.5	5.9	Presumed	Type I pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Minimal reactivity to handling	2,4-D	< 5	5	Unknown	Cell and mitochondrial membrane damage, uncoupling of oxidative phosphorylation and interference of cellular metabolism?
	Negative air drop, pupillary responses, decreased analgesic reflex	Ethoprophos	2.65	27.11		Inhibition of acetylcholinesterase (AChE)
	No reaction to noise	Metaldehyde	30	90	Presumed	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?
	Retarded pupillary reflex	Fenpropimorph	7.1	71	Unknown	
	Sensory changes (proprioceptive deficit)	Propineb	4.3	41.4	Unknown	Actin depolymerisation, disruption of cytoskeletal organisation and further acetylcholine release.
	Sensory changes (presthesia, proprioception deficit)	Molinate	10	50	Unknown	Inhibition of aldehyde dehydrogenase and further accumulation of endogenous neurotoxic metabolites of neurotransmitters (e.g. dopamine)
Functional effects on autonomic division	Lacrimation	Oxamyl	0.1	1.5	Known	Inhibition of acetylcholinesterase (AChE)
	Lacrimation	tau-Fluvalinate	0.5	1	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Lacrimation	Ethoprophos	2.65	27.11	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Lacrimation	Tri-allate	32	177	Unknown	
	Mydriasis	Abamectin	0.25	0.5	Presumed	Binding to GABA receptors and GABA gated ion channels
	Mydriasis	Deltamethrin	1	2.5	Presumed	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Piloerection	Fluquinconazole	1.73	8.81	Unknown	
	Piloerection	Lambda-Cyhalothrin (study performed with Cyhalothrin)	1.8	9.2	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Piloerection	Tefluthrin	11.6	26.6	Presumed	Type I pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Piloerection	Triadimenol (a metabolite of Triadimefon)	40	209	Unknown	Increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways?
	Salivation	Methiocarb	0.05	0.5	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Abamectin	0.25	0.5	Presumed	Binding to GABA receptors and GABA gated ion channels
	Salivation	tau-Fluvalinate	0.5	1	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Salivation	Molinate	1	10	Unknown	Inhibition of aldehyde dehydrogenase and further accumulation of endogenous neurotoxic metabolites of neurotransmitters (e.g. dopamine)
	Salivation	Lambda-Cyhalothrin (study performed with Cyhalothrin)	2.5	10	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Salivation	Ethoprophos	2.65	27.11	Known	Inhibition of acetylcholinesterase (AChE)
	Salivation	Lufenuron	3.64	29.8	Unknown	

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Salivation	Chlormequat	5	10	Unknown	Partial agonist of the nicotinic acetylcholine receptor (nAChR)
	Salivation	Cypermethrin	6	20	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Salivation	Clothianidin	19.3	40.9	Known	Nicotinic acetylcholine receptor (nAChR) agonist
	Salivation	Metaldehyde	30	90	Presumed	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?
	Salivation	Dicamba	50	300	Unknown	Disruption of cell metabolism, uncoupling of oxidative phosphorylation?
	Salivation	Carbetamide	150	300	Unknown	
	Salivation	Mepiquat	< 166	166	Presumed	Activation of nicotinic and muscarinic acetylcholine receptors
	Trismus salivation	Glufosinate	4.5	8.4	Presumed	Inhibition of glutamine synthetase and interference of nitrogen metabolism
	Urination	Indoxacarb	2.6	14	Presumed	Presumed blocking of sodium channels (in insects)
	Urination	Pirimicarb	10	25	Known	Inhibition of acetylcholinesterase (AChE)
	Urination	2,4-D	75	150	Unknown	Cell and mitochondrial membrane damage, uncoupling of oxidative phosphorylation and interference of cellular metabolism?
Neurochemical end-points	AChE inhibition (brain)	Pirimiphos-methyl	0.25	> 0.25	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Phosmet	< 1	1	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Methomyl	9	95	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (brain)	Pirimicarb	10	25	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	AChE inhibition (erythrocytes)	Ethoprophos	0.025	1	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Chlorpyrifos	0.03	0.1	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Dimethoate	0.04	0.2	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Fenamiphos	0.042	0.15	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Methiocarb	< 0.05	0.05	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Chlorpyrifos-methyl	0.1	3.9	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Formetanate	0.37	1.75	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Pirimiphos-methyl	0.4	2	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Fosthiazate	0.48	0.97	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Oxamyl	1.69	15.3	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Malathion	3	35	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Pirimicarb	10	25	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Ethephon	13	66	Known	Inhibition of acetylcholinesterase (AChE)
	AChE inhibition (erythrocytes)	Methomyl	41	55	Known	Inhibition of acetylcholinesterase (AChE)
Neuropathological end-points	Axonal degeneration	Flufenacet	1.14	27	Presumed	Metabolic lesions due to limitations in glutathione interdependent pathways and antioxidant stress

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Axonal degeneration	Oxasulfuron	1.5	99	Unknown	
	Axonal degeneration	Molinate	1.8	13	Unknown	Inhibition of aldehyde dehydrogenase and further accumulation of endogenous neurotoxic metabolites of neurotransmitters (e.g. dopamine)
	Axonal degeneration	Indoxacarb	4	20	Presumed	Presumed blocking of sodium channels (in insects)
	Axonal degeneration	Cymoxanil	5	38	Unknown	
	Axonal degeneration	Tri-allate	6.4	32	Unknown	
	Axonal degeneration	Ziram	9	27	Presumed	Neurotoxic effect might be due to the metabolite CS_2
	Axonal degeneration	Metaldehyde	10	50	Presumed	Decreased brain concentration of neurotransmitters (GABA, NE, 5-HT)?
	Axonal degeneration	tau-Fluvalinate	< 10	10	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Axonal degeneration	Isoxaflutole	20	500	Unknown	
	Axonal degeneration (degeneration of trigeminus and increased galactosidase activity)	Cypermethrin	25	50	Unknown	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Cerebral cortex vacuolisation	Sulfuryl fluoride	1.4	5.6	Unknown	
	Encephalomalacia (vacuolisation)	Tetraconazole[1]	< 0.5	0.5	Unknown	Neurotoxic potential of 1,2,4-triazole, a metabolite common to a number of triazole-derivative pesticides

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Myelin degeneration (secondary to axonal degeneration)	Oxasulfuron	1.5	99	Unknown	
	Myelin degeneration	Molinate	1.8	13	Unknown	Inhibition of aldehyde dehydrogenase and further accumulation of endogenous neurotoxic metabolites of neurotransmitters (e.g. dopamine)
	Myelin degeneration	Quinoclamine	3.82	40.2	Unknown	
	Myelin degeneration	Cymoxanil	5	38	Unknown	
	Myelin degeneration	Fenpropidin	5	20	Unknown	Disturbance of cholesterol supply/synthesis by Schwann cells
	Myelin degeneration	Tri-allate	6.4	32	Unknown	
	Myelin degeneration (myelin damage and Schwann cell proliferation)	Mancozeb	8.2	49	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)
	Myelin degeneration	tau-Fluvalinate	< 10	10	Known	Type II (α-cyano) pyrethroid. Delaying of the closing of sodium channels in the nervous system
	Myelin degeneration (a treatment-related exacerbation of spontaneous age-related	Pirimicarb	15.6	47.4	Known	Inhibition of acetylcholinesterase (AChE)

CAG 2	Indicator of specific effect	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	changes?)					
	Myelin degeneration	Isoxaflutole	20	500	Unknown	
	Myelin degeneration	Pyridate	60	200	Unknown	
	Neuronal degeneration/ necrosis	Indoxacarb	4	20	Presumed	Presumed blocking of sodium channels (in insects)
	Sciatic nerve lesions (not specified)	Thiram	1.4	14	Unknown	Neurotoxic effect might be due to the metabolite CS_2 (common metabolite of dithiocarbamates)

[1] Substance excluded by DTU

E. BACKGROUND FOR HAZARD IDENTIFICATION AND CHARACTERISATION FOR EFFECTS ON THE THYROID SYSTEM

Considerations on elaboration of CAGs for substances affecting the thyroid or thyroid hormone system.

1. CAG level 1

In the DTU report (2012), CAG level 1 was defined as "Toxicity to the thyroid gland". A number of major specific effects identified by DTU concern the populations of calcitonin-producing parafollicular cells (C-cells) and thyroid hormone (T3/T4)-producing follicular cells. Both of these cell populations secrete specific hormones which act systemically. At least for the thyroid hormone system involving T3/T4, changes in serum hormone levels may not only result from toxicity to the thyroid itself, but may be due to mode of action operating outside the thyroid (e.g. enzyme induction in the liver). Consequently, it appears sensible to expand the CAG level 1 to actually comprise not only toxicity to the thyroid gland itself, but also to the hypothalamic-pituitary-thyroid axis (affecting function of thyroid hormones and calcitonin).

2. CAG level 2

The evaluation summarised in section 8.1. for the thyroid distinguishes between specific effects concerning the parafollicular cells and specific effects concerning the thyroid follicular cells or the thyroid hormone system. This is in line with the general distinction made within the DTU report.

2.1. CAG 2A: Substances affecting the thyroid parafollicular cells (C-cells) or the calcitonin system

It is noted that information on serum levels of calcitonin, a hormone that plays a role in calcium homeostasis, is generally not available from toxicological studies. However, concerning the calcitonin-producing C-cells, two specific effects were identified: C-cell hyperplasia/hypertrophy and C-cell neoplasia. Since C-cell stimulation leading to cell proliferation and hyperplasia is expected to play a promoting role in further progression to neoplasia, it is proposed to combine substances displaying either or both of these effects into one phenomenological level 2 CAG. In the absence of specific knowledge on the underlying modes of action for pesticide active substances leading to C-cell activation/proliferation, further subgrouping is not recommended.

2.2. CAG 2B: Substances affecting the thyroid follicular cells or the thyroid hormone system

2.2.1. General considerations

Chemicals disrupting the thyroid hormone (TH) system may be broadly defined as "xenobiotics that interfere with TH signalling" (Miller et al., 2009), including xenobiotics that alter the structure or function of the thyroid gland, affect enzymes associated with thyroid hormone (TH: T3 or T4) homeostasis, change circulating or tissue concentrations of thyroid hormones or alter binding of thyroid hormones to cellular receptors (Miller et al., 2009; Crofton et al., 2005). Accordingly, perturbations of the thyroid hormone system may result in manifestations concerning the thyroid itself (e.g. histological changes, thyroid enlargement, thyroid tumours), or effects regarding non-thyroid TH-dependent tissues.

The following figure 1 (from Miller et al., 2009) illustrates pathways controlling thyroid hormone homeostasis and possible targets for thyroid hormone system disruption. A variety of modes/mechanisms of action that may play a role in disruption of the thyroid hormone system have also been addressed in the DTU Report.

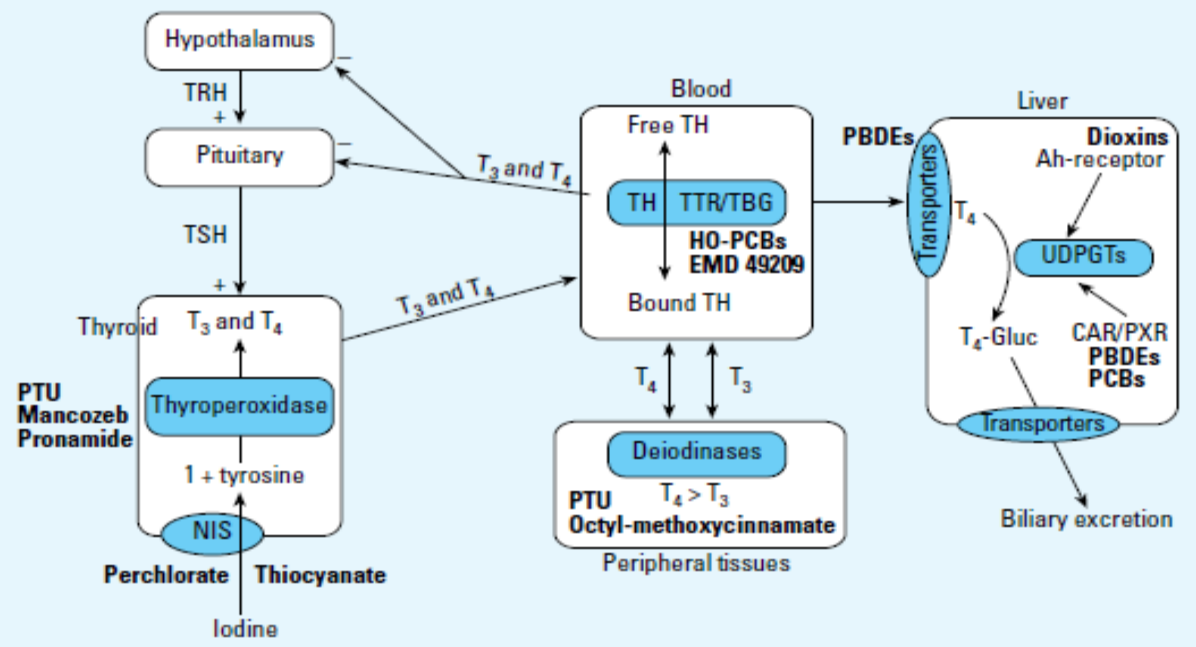

Figure 1: Thyroid hormone system with potential targets for disruption by xenobiotics (blue). NIS, sodium/iodide symporter; TBG, thyroid hormone-binding globulin; TH, thyroid hormone (T3/T4); T3, triiodothyronine; T4, thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; TTR, transthyretin; UDPGTs, UDP-glucuronosyl transferases (Miller et al., 2009).

As a consequence of changes in circulating and tissue thyroid hormone levels, compensatory mechanisms including activation of the hypothalamic-pituitary-thyroid axis following a decline in peripheral thyroid hormone levels with subsequent increased production and secretion of TSH (thyroid stimulating hormone) may be expected to result in adjustment of bioavailable thyroid hormone levels, rendering changes in circulating or tissue T3/T4 hormone levels transient.

2.2.2. Relevance of thyroid hormone system disruption to humans

Concerning effects on the thyroid itself, prolonged enhanced secretion by the pituitary of TSH as a response to decreased circulating thyroid hormone levels in rat studies leads to thyroid follicular cell hypertrophy and hyperplasia, which eventually may act as a promoting factor in the development of benign and malignant follicular cell tumours. Although compensatory mechanisms based on feedback loops within the hypothalamic-pituitary-thyroid axis are also operative in humans from a qualitative point of view, it appears that humans are quantitatively less susceptible to follicular cell tumour formation resulting from thyroid hormone system imbalance than rats, based on marked quantitative differences in kinetics of circulating thyroid hormones and in the extent of response to changes in thyroid hormone levels (Dellarco et al., 2006). Nevertheless, alterations in animal studies such as thyroid hypertrophy and hyperplasia may also be seen as general indicators of disturbance of thyroid hormone homeostasis or of preceding changes in systemic thyroid hormone levels. Alterations in circulating bioavailable thyroid hormone levels may have serious impact on other organs or organ systems besides the thyroid itself also in humans, particularly if perturbations occur during critical windows of development.

The DTU report lists a number of reasons why humans may be less sensitive to perturbations of the thyroid hormone system than rats. It appears plausible that the healthy human adult individual displays a certain propensity for coping with challenges to the thyroid hormone system, owing to buffering mechanisms (e.g. plasma protein binding of thyroid hormones, storage of thyroglobulin in follicular colloid), and to the presence of various compensatory mechanisms directed at reestablishment of

thyroid hormone homeostasis. Nevertheless, epidemiological studies suggest that higher TSH levels (as an indicator of challenges to thyroid hormone homeostasis and subsequent activation of the hypothalamic-pituitary-thyroid axis), even in the context of subclinical hypothyroidism, are associated with increased risks for adverse organ effects in adults, e.g. regarding cardiovascular endpoints (reviewed in Miller et al., 2009).

There are indications that the developing child is particularly sensitive to perturbations of the thyroid hormone system, in that an inadequate supply of thyroid hormones during critical windows of development may severely impact development or even lead to irreversible effects. Before onset of fetal thyroid function during the second trimester of pregnancy, placental transfer of maternal thyroid hormones appears to represent the sole source of thyroid hormone to the developing child (Gilbert et al., 2012). Even in more advanced stages of pregnancy, maternal thyroid hormones are thought to contribute to adequate fetal supply. In addition, very limited storage capacity for thyroid hormone appears to exist for humans during the fetal, newborn and early infant stages in comparison to extensive storage within the thyroid follicular colloids in adults (reviewed in Woodruff et al., 2008), and T4 appears to also display a shorter half-life as compared to adults, pointing to a higher susceptibility also on a kinetic basis towards effects resulting from thyroid hormone imbalance.

Studies regarding the outcome of human pregnancies in respect to maternal thyroid function provide evidence that even moderate or transient thyroid hormone deficiency during critical periods of development may affect among others neurodevelopmental endpoints such as intelligence of the child (Haddow et al., 1999; Morreale de Escobar et al., 2000; Kooistra et al., 2006). For example, infants of seemingly healthy mothers with maternal free T4 serum levels below the 10th percentile during the first trimester showed significantly lower scores on a neonatal behavioral assessment orientation index (Kooistra et al., 2006). In conclusion, even transient changes in T3 and T4 levels in humans, depending on the duration, timing and extent of relative T3 and T4 insufficiency, may affect the developing nervous system of the unborn child.

Thyroid hormone homeostasis may not only be affected by exposure to man-made xenobiotics as individual substances or as mixtures, but is also influenced by other dietary or lifestyle factors, e.g. extent of iodine ingestion, exposure to inhibitors of thyroid hormone production such as thiocyanate via ingestion of cruciferous vegetables or smoking (Steinmaus et al., 2007). In addition, the basal functional state of the thyroid may already be challenged due to individual biological processes (pregnancy) or compromised in disease conditions (e.g. autoimmune thyroiditis). Thus, variability in the population concerning susceptibility towards additional chemical insults affecting the thyroid hormone system can be assumed.

2.2.3 Phenomenological effects regarded as being relevant for assignment to cumulative assessment groups

For the evaluation of the common toxicological profile for assignment of an active substance to a CAG, different indicators may be taken into account, which could comprise downstream endpoints with obviously adverse target organ effects or upstream precursor effects, e.g. a decrease in T4 levels, that may eventually lead to manifestation of an adverse organ effect.

Based on the assumption that the developing child is particularly sensitive to changes in circulating hormone levels that may still be tolerated by the mother, but also the epidemiological evidence for adults linking even subclinical hypothyroidism to increased risk for cardiovascular disease, it has recently been concluded that:

"…TDC [thyroid disrupting chemical] exposures that would result in reduced T4 in a population should be considered an adverse effect" (Woodruff et al., 2008) and that

"Any degree of thyroid disruption that lowers TH levels on a population basis should be considered a biomarker of increased risk of adverse outcomes, which may have important societal outcomes" (Miller et al., 2009).

In the context of CRA, it is therefore proposed to also consider the physiological change preceding adverse manifestations in target organs (changes in circulating thyroid hormone levels) and indicators of perturbation of thyroid hormone homeostasis (e.g. elevation of TSH or thyroid enlargement), to be of relevance for definition of cumulative assessment groups. This approach has been followed in the DTU report.

2.2.4 Specific effects and indicators leading to assignment to a common CAG2B:

The following specific effects were identified by DTU for the thyroid and confirmed in the course of the review of the report as being related to the T3/T4-associated thyroid hormone system:

- Changes in serum T3 and/or T4 (in rare cases there was information on total vs. free hormone)
- Changes in serum TSH
- Follicular cell hyperplasia/hypertrophy and/or increased thyroid weight
- Follicular cell tumours

These effects may be used for screening of toxicological databases and allocation of substances to CAG2B. These effects are interrelated and connected by a chain of events with one another, even if detailed mechanistic information is not available. This was supported by the comparison of different single effect groups that were documented in the DTU report, in that the tentative single effect groups displayed considerable overlap with one another. For example, in our data collection, all substances fulfilling the single phenomenological criterion "TSH increase" also affected serum T3 and/or T4 levels, with the exception of three, which, however, did lead to follicular cell hyperplasia/hypertrophy. Almost all substances displaying an increase in TSH and most of the substances affecting T3/T4 levels according to the DTU report also led to follicular cell hypertrophy/hyperplasia or thyroid weight increase. Finally, the preliminary group based on thyroid follicular tumours was actually a subgroup of the "Follicular cell hyperplasia/hypertrophy" effect group. As stated in the DTU report:

"As already described under CAG level 2, the different effects on the thyroid follicular cells or on the thyroid hormone levels are often not independent effects, but rather consequences of each other. The combined mode of action model illustrates that many different mechanisms of action may lead to serum T3 and T4 changes. Subsequently, TSH is increased to compensate for the decreased T3 and/or T4. Persistent elevation of TSH may lead to histopathological changes such as thyroid follicular cell hypertrophy and even thyroid follicular cell tumours (at least in rodents)."

In conclusion, the single effects listed above may serve as indicators or surrogates for each other. Consequently, substances displaying at least one of the above listed effects would be allocated to the combined phenomenological CAG2B. In addition to the specific effects mentioned above, the DTU report identified inflammation of the thyroid and/or degeneration of the thyroid gland as a general mode of action leading to damage of follicular cells. Thyroid inflammation, and possibly also other processes leading to degenerative changes in follicular cells, may be expected to affect thyroid function, resulting in changes in T3/T4 or TSH levels. It was therefore concluded by the Panel that substances adversely affecting thyroid function via inflammation and/or degeneration should be included in CAG2B. Thus, in the process of data collection, effects of thyroid inflammation and follicular cell degeneration, if clearly shown to be substance-related, should be considered as alerts for other effects concerning thyroid function. Additional histopathological changes reported in DARs and listed in the DTU report, such as increased/decreased amount of colloids, small/large follicles,

different shapes of follicular cells, increased vascularisation, increased vacuolisation, follicular cysts, follicular cell pigmentation, follicular atrophy or necrosis of follicular cells were regarded as being covered by the combined CAG2B.

It is noted that, although e.g. the new extended one generation study (OECD, 2012) contains provisions for measurement of thyroid hormones or TSH, standard OECD guidelines for repeated dose toxicity testing do not include hormone determinations as a mandatory requirement. This may pose a limitation in terms of availability of information on hormone levels and thus theoretically impede use of thyroid functional parameters for allocation to CAG2B. However, histopathological evaluations of the thyroid are performed in the standard repeated-dose toxicity studies, and thus an information gap concerning hormone levels can be expected to be largely compensated for by other specific effects associated with CAG2B.

In preparation for CRA, the NOAELs from the specific effects determining the allocation of an individual substance to CAG2B would be compared to define the most sensitive relevant NOAEL to be used for CRA.

2.2.5 Mechanistic considerations concerning substances in CAG2B - General mode of action: Toxicity to thyroid follicular cells or to the T3/T4 system

A number of pesticide active substances may influence the thyroid system through one or more mechanisms. Based on the interrelationship between different targets within the thyroid hormone system, there may be concern that combinations of individual substances potentially affecting the thyroid hormone system may feed into common processes which in the end may result in impairment of effector hormone- (basically T3-) dependent receptor signalling.

A list of mode of actions affecting the thyroid or thyroid hormone system has been summarized within the DTU report. Figure 2 shows a scheme, based on the review by Miller et al., 2009, which outlines several general toxicological targets within the thyroid hormone system. It refers to both thyroidal and extrathyroidal targets and illustrates that different modes/mechanisms of action may contribute to a common clinical outcome, e.g. impairment of brain development. A similar scheme is also presented in the DTU report. In addition to the scheme in the DTU report, interference with binding of thyroid hormones to circulating blood proteins is also considered as a potentially relevant mode of action. Displacement of T4/T3 from plasma protein would be expected to contribute to a shorter half-life of circulating hormone.

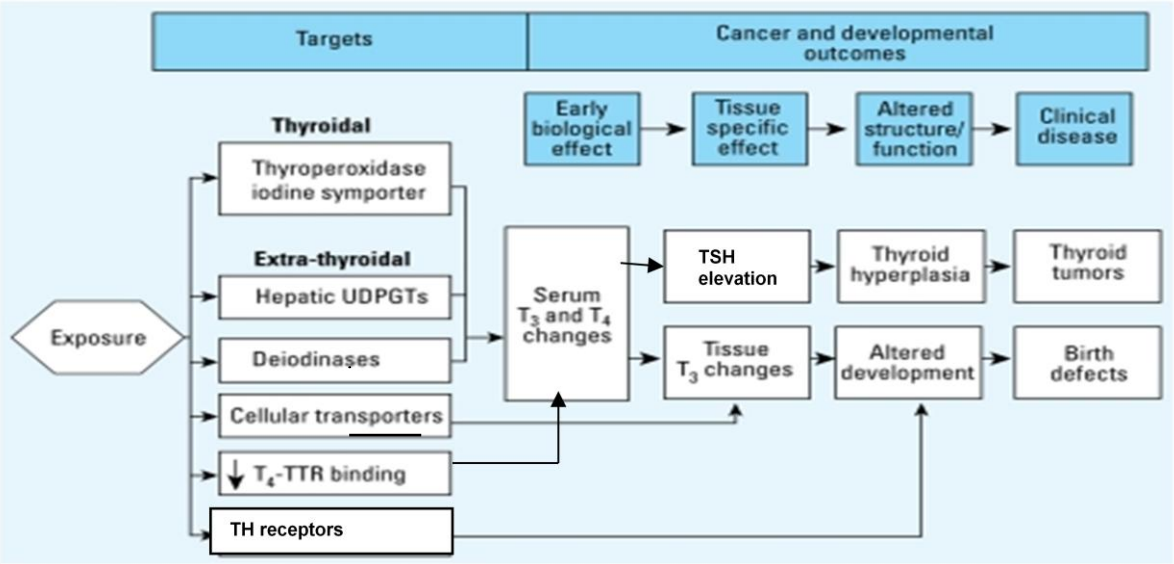

Figure 2: Examples of targets of thyroid hormone (T3/T4) system disruption and potential outcomes.

T3, triiodothyronine; T4, thyroxine; TH, thyroid hormone; TTR, transthyretin (major T4-binding protein in rats); UDPGT, UDP-glucuronosyl transferases. Modified from Miller et al., 2009.

In the EFSA Opinion and Triazole exercise (2008, 2009) it was advised that, starting from a group of chemicals producing the same toxic effect, further refinement of grouping can be achieved based on the mode/mechanism of action of each individual substance under consideration of the key events involved.

Although most substances affecting the thyroid follicular cells or the thyroid hormone system appear to generally be associated with a decrease in thyroid hormone (T3/T4) levels or ultimate thyroid hormone action, there may be substances that stimulate thyroid or enhance thyroid hormone release. Theoretically, specific mechanistic information might be used to consider exclusion of a substance from the combined CAG2B group.

Apart from the above mentioned exception, a further sub-grouping of CAG2B based on different modes/mechanisms of action is currently not recommended. The reasons for this conclusion are detailed below.

- For many substances affecting the thyroid or the thyroid hormone system on an effect basis, the mechanism of action has not been defined. From a conservative point of view, such substances might be considered for inclusion in CRA if indicators for perturbation of thyroid hormone homeostasis have been observed. This is in accordance with the recommendations received from the EU Commission.

- In particular, the degree of similarity or dissimilarity of various modes/mechanisms of action may be difficult to define or to agree upon in studies concerning mixtures of chemicals affecting the thyroid hormone system. For example, different chemicals may affect thyroid hormone clearance via a mode of action related to enzyme induction, yet individual substances may convey this induction via different cellular pathways (activation of the Aryl hydrocarbon (Ah) receptor vs. activation of nuclear receptors such as CAR or PXR) and may thus modulate expression of distinct patterns of enzymes involved in thyroid hormone metabolism.

- Some substances may act via several mechanisms, e.g. enzyme induction, displacement from plasma protein binding, interference with thyroid hormone receptor-dependent transactivation (Miller et al., 2009).

- In the paper by Crofton et al., 2005, individual substances affecting thyroid hormone clearance via different molecular mechanisms and their mixtures were tested in rats over a short-term exposure period of 4 days. Mixtures were tested at several doses, the highest dose levels being below or corresponding to individual NOELs. It was reported that the cumulative effect of the mixture was predicted by dose-dependent additivity at low doses and synergism at high doses. More precisely, the mixture dose-response curve in the lower dose range (up to about a factor of 10 lower than the highest concentration tested) was in accordance with the dose-dependent additivity prediction, although this was the non-linear part of the dose response, in which it is difficult to discern any changes in the response. With higher concentrations of the mixture however, the observed response was in accordance with synergism, and was underpredicted by the dose-additivity model. Noteworthy is the fact that the dose-dependent additivity model up to the individual NOELs would have been less underpredictive than an effect addition prediction. According to the latter, no response towards the mixture would be expected if all components are below their individual effect levels. In a more recent paper (Flippin et al., 2009) rats were treated with dilutions of a mixture of 21 substances, containing both stimulators of T4 clearance in the liver and substances affecting TH synthesis. Predictive modelling was performed, comparing three additivity model predictions (dose addition, effect addition and integrated addition). It was found that both dose and integrated addition models provided similar results, with better predictions than the effect-addition model. Consequently, in the DTU report, it is concluded that: “These two studies suggest that it may be possible to predict a decreased level of T4 in a mixture of chemicals with a fair degree of accuracy using

dose addition models without knowing the detailed mechanism behind the decreased T4 level".

- Ultimately, downstream organ or tissue effects will be based on the interaction or perturbation of interaction of the effector hormone (predominantly T3) on the receptor level. Different mechanisms outlined in the DTU report may principally affect processes expected to be integrated concerning the extent of thyroid hormone action.

F. DATA COLLECTION FOR EFFECTS ON THE THYROID SYSTEM

F.1. Data collection for thyroid toxicity- EFSA Part 1

See separate file.

F.2. Data collection for thyroid toxicity – EFSA Part 2

See separate file.

G. CAGs FOR THE THYROID SYSTEM

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
CAG2A	C-cell adenoma	Quizalofop-P-tefuryl	1.7	48.7	Unknown	
Effects on the parafollicular (C-) cells or the calcitonin system	C-cell adenoma	Mepanipyrim	<2.45	2.45	Unknown	
	C-cell adenoma	Fenbuconazole	3	31	Unknown	
	C-cell adenoma	2,4-DB	<3	3	Unknown	
	C-cell adenoma	Bromuconazole	6.48	43.3	Unknown	
	C-cell adenoma	Thiram	7.31	14.66	Unknown	
	C-cell adenoma	Oryzalin	12	36	Unknown	
	C-cell adenoma	Hexythiazox	29.3	207	Unknown	
	C-cell adenoma / carcinoma	Oxyfluorfen	1.94	57	Unknown	
	C-cell adenoma / carcinoma	Lenacil	16	160	Unknown	
	C-cell adenoma / carcinoma	Penoxsulam	50	250	Unknown	
	C-cell hyperplasia	Amitrole (aminotriazole)	0.3	13	Unknown	
	C-cell hyperplasia	Fenamidone	2.8	7.1	Unknown	
	C-cell hyperplasia	Desmedipham	3.18	15.71	Unknown	
	C-cell hyperplasia	Dichlorprop-P	<3.5	3.5	Unknown	
	C-cell hyperplasia	Ziram	7.7	23.7	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	C-cell hyperplasia	Buprofezin	8.71	89.5	Unknown	
	C-cell hyperplasia	Imidacloprid	51.3	102.6	Unknown	
	C-cell hyperplasia	2,4-D	75	150	Unknown	
	C-cell hyperplasia	Folpet	1800	4000	Unknown	
	C-cell hyperplasia / adenoma / carcinoma	Dodine	20	42	Unknown	
	C-cell neoplasia	Ioxynil	0.6	1.8	Unknown	
CAG2B Substances affecting follicular cells and/or thyroid hormone (T3/T4) system	Decreased circulating T3 and/or T4 levels	Fipronil	<0.02	0.02	Known	Enhanced T4 clearance from blood, enhanced biliary clearance
	Decreased circulating T3 level / follicular cell adenoma / carcinoma	Ioxynil	<0.2	0.2	Presumed	Hepatic enzyme induction
	Decreased circulating T3 and T4 levels	Bromoxynil	<0.9	0.9	Unknown	
	Decreased circulating T4 and T3 levels / increased circulating rT3 level / follicular hypertrophy / follicular hyperplasia	Proquinazid	1.2	12	Presumed	Induction of UDP-glucuronosyl transferase, leading to decreased half-life of T4 and increase in TSH. An increase in rT3 and decrease in T3 may be in line with decreased activity of hepatic 5´-deiodinase
	Decreased circulating T3 and/or T4 levels	Flufenacet	1.7	6.9	Presumed	Increased T4 metabolism
	Decreased circulating free T3/T4 level / follicular cell hypertrophy / increased relative thyroid weight	Haloxyfop-P (Haloxyfop-R)	2	5	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Decreased circulating T3 and T4 levels	Dithianon	< 2.5	2.5	Unknown	
	Decreased circulating T4 levels	Desmedipham	<3.86	3.86	Unknown	
	Decreased circulating T3 and T4 levels / follicular cell hyperplasia	Metiram	5	15	Known	Inhibition of thyroid peroxidase (metabolite ETU)
	Decreased circulating T3 level	Fluoxastrobin	3	24	Presumed	Hepatic enzyme induction
	Decreased circulating T3 level	Pyrethrins	<6.6	6.6	Presumed	Hepatic enzyme induction
	Decreased circulating T4 level	Lufenuron	7	30	Unknown	
	Decreased circulating T3 level	Lenacil	<22.5	22.5	Unknown	Pigmentation at higher doses in rats indicates accumulation in follicular cells
	Decreased circulating T4 level	Pyridate	177	500	Unknown	
	Disappearance of thyroid colloid	Formetanate	<5	5	Unknown	
	Mineralisation in follicle colloid (reflecting premature aging of the thyroid)	Imidacloprid	5.7	16.9	Unknown	
	Enlarged thyroid	Ethofenprox	1.1	3.7	Known	Liver enzyme induction
	Enlarged thyroid / follicular cell hypertrophy	Clodinafop	10.2	26.3	Unknown	
	Enlarged thyroid / follicular cell hyperplasia / follicular cell adenoma	Pethoxamid	17	70	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Follicular cell adenoma	Cyhalofop-butyl	0.1	0.2	Unknown	
	Follicular cell adenoma / carcinoma	Oxadiargyl	2.1	21.5	Unknown	
	Follicular cell adenoma	Diclofop	2.25	22.5	Unknown	
	Follicular cell adenoma /carcinoma	Etridiazole	5	30	Unknown	
	Follicular cell adenoma	Thiabendazole	10	30	Presumed	Hepatic enzyme induction
	Follicular cell adenoma / follicular cell carcinoma	Diethofencarb	42.7	220.3	Known	Increased T4-UDP-glucuronosyl transferase activity
	Follicular cell adenoma / adenocarcinoma	Penoxsulam	50	250	Unknown	
	Follicular cell carcinoma	Mepanipyrim	<2.45	2.45	Unknown	
	Follicular cell hyperplasia / adenocarcinoma	Fluquinconazole	0.44	4.77	Unknown	
	Follicular cell hyperplasia / follicular cell adenoma	Isoxaflutole	2	20	Presumed	Result of hepatic effects
	Follicular cell hyperplasia	Maneb	3.7	14	Known	Inhibition of thyroid peroxidase (by metabolite ETU); inhibition of iodide uptake
	Follicular cell hyperplasia	Terbuthylazine	6.97	41.5	Unknown	
	Follicular cell hyperplasia / adenoma	Propyzamide	8.5	42.6	Presumed	Heptic enzyme induction (increased biliary clearance in 15-week rat study)
	Follicular cell hyperplasia	Benthiavalicarb	9.9	249	Presumed	Pigmentation in mouse follicular cells indicates accumulation of substance in follicular cells.
	Follicular cell hyperplasia / adenoma / carcinoma	Oryzalin	36	111	Unknown	Unknown for rats; reduced T4 protein binding considered for dogs
	Follicular cell hyperplasia /	Silthiofam	50.5	149.8	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	adenoma / carcinoma					
	Follicular cell hyperplasia	Folpet	68.4	224	Unknown	
	Follicular cell hyperplasia	Hymexazol	98	292	Unknown	
	Follicular cell hypertrophy	Propineb	0.18 (overall NOEL)	0.9	Presumed	Inhibition of thyroid peroxidase
	Follicular cell hypertrophy	Ziram	0.56	5.5	Unknown	
	Follicular cell hypertrophy /hyperplasia	Buprofezin	0.9	8.7	Presumed	Increased metabolism of T4/(T3) in liver
	Follicular cell hypertrophy	Tetraconazole	1	8.3	Presumed	Hepatic enzyme induction; no inhibition of deiodinase activity converting T4 to T3
	Follicular cell hypertrophy	Thiacloprid	1.2	2.5	Presumed	Hepatic enzyme induction
	Follicular cell hypertrophy	Quizalofop-P-tefuryl	1.3	39.5	Presumed	Hepatic enzyme induction
	Follicular cell hypertrophy / hyperplasia	Picolinafen	1.7	12.5	Unknown	
	Follicular cell hypertrophy	Cyprodinil	3.14	19	Unknown	
	Follicular cell hypertrophy	Aclonifen	3.6	35.4	Unknown	
	Follicular cell hypertrophy / hyperplasia	Fenamidone	3.6	7.1	Presumed	Results of effects occurring in liver
	Follicular cell hypertrophy / decreased circulating T4 level	Mancozeb	4	17	Known	Inhibition of thyroid peroxidase (by metabolite ETU); inhibition of iodide uptake
	Follicular cell hypertrophy	Fenbuconazole	5.7	28	Presumed	Liver enzyme induction leading to increased hormone clearance
	Follicular cell hypertrophy	Dinocap	10	100	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Follicular cell hypertrophy	Fenoxycarb	10.1	49.6	Unknown	
	Follicular cell hypertrophy / hyperplasia / pigmentation	Pyrimethanil	17	221	Presumed	Hepatic enzyme induction
	Follicular cell hypertrophy / decreased circulating T4 level	Oxadiazon	17.8	62.1	Unknown	
	Follicular cell hypertrophy	Carbetamide	20.1	150.3	Presumed	Pigmentation of follicular cells in rats indicates substance accumulation in the thyroid
	Follicular cell hypertrophy	Amidosulfuron	23.7	121.2	Unknown	
	Follicular cell hypertrophy	Cyproconazole	24.7	52.8	Presumed	Hepatic enzyme induction
	Follicular cell hypertrophy / hyperplasia	Fluopicolide	32	109	Presumed	Hepatic enzyme induction
	Follicular cell hypertrophy / pigmentation	Pendimethalin	43	88	Known	Hepatic enzyme induction; (presumed: pigmentation indicates accumulation of substance in thyroid follicular cells)
	Follicular cell hypertrophy / hyperplasia	Maleic hydrazide	63	625	Unknown	
	Follicular cell hypertrophy	Imazosulfuron	75	150	Unknown	
	Folllicular cell hypertrophy / hyperplasia	Benalaxyl	100	800	Unknown	
	Follicular cell hypertrophy	Thiamethoxam	198.6	710.6	Presumed	Hepatic enzyme induction
	Follicular cell hypertrophy	Zoxamide	281	1054	Unknown	
	Increased circulating T4 level	Clofentezine	1.73	17.3	Presumed	Increased hepatic metabolism; circulating TSH level elevated in rat studies
	Increased circulating TSH/T4 level	Spirodiclofen	14.72	110.14	Unknown	(Both T4 and TSH increase were observed in rat at higher doses.)
	Decreased circulating T4	Prothioconazole	5	50	Presumed	Hepatic enzyme induction

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	level					
	Increased relative thyroid weight	Amitrole (aminotriazole)	0.3	1	Known	Inhibition of thyroid hormone synthesis (Inhibition of thyroid peroxidase, inhibition of iodide uptake into follicular cells)
	Increased thyroid weight	MCPA (metabolite of MCPB)	0.95	9.3	Unknown	(MCPA-thioethyl)
	Increased relative thyroid weight	2,4-D	1	5	Unknown	
	Increased relative thyroid weight	Dazomet	1	3.1	Unknown	
	Increased relative thyroid weight / follicular cell adenoma	Oxyfluorfen	1.94	57	Unknown	
	Increased relative thyroid weight	Spinosad	2.7	8.2	Presumed	Inflammation of thyroid, vacuolization of follicular cells which is consistent with phospholipidosis (Yano et al., 2002). Vacuolisation occurring also in other organs (i. a. liver, kidney, adrenals)
	Increased relative thyroid weight	Quinoclamine	2.9	38.3	Unknown	
	Increased relative thyroid weight	Bupirimate	3	15	Presumed	Increased metabolism of T4 /(T3) and excretion into the bile; inhibition of thyroid hormone synthesis
	Increased thyroid weight	Propaquizafop	3	15	Unknown	
	Increased relative thyroid weight	Fuberidazole	3.6	18	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Increased relative thyroid weight / decreased circulating T3 and T4 levels	Chlorprofam	5	50	Unknown	
	Increased relative thyroid weight / follicular cell hyperplasia / adenoma/ carcinoma	Benfluralin	5.4	136	Unknown	
	Increased relative thyroid weight	Bromuconazole	6.48	87.2	Unknown	
	Increased relative thyroid weight / decreased circulating T4 level	Thiophanate-methyl	8	40	Unknown	
	Increased relative thyroid weight	Carboxin	12	37	Unknown	
	Increased relative thyroid weight	Tepraloxydim	14	66	Prsumed	Hepatic enzyme induction
	Increased relative thyroid weight	Myclobutanil	15	51.5	Unknown	
	Increased thyroid weight	Tribenuron (aka metometurun)	15	73	Unknown	
	Increased relative thyroid weight / follicular cell adenoma	Beflubatamid	17.7	150	Unknown	
	Increased thyroid weight	Flumioxazin	19.3	90	Unknown	
	Increased relative thyroid weight	Cyflufenamid	20	120	Presumed	Enhanced hepatic metabolism
	Increased relative thyroid weight	Boscalid	22	57	Unknown	
	Increased relative thyroid weight	Prochloraz	25	100	Unknown	
	Increased relative thyroid weight	Tolylfluanid	33	93	Unknown	

CAG2	Indicator of specific effect (only most sensitive indicator(s)) observed)	Active substance	NO(A)EL mg/kg bw per day	LO(A)EL mg/kg bw per day	Mode/mechanism of action	
	Increased relative thyroid weight	Flutolanil	37	299	Unknown	
	Increased relative thyroid weight	Cycloxydim	50	250	Unknown	
	Increased relative thyroid weight	Clethodim	62	250	Unknown	
	Increased relative thyroid weight	Tritosulfuron	92	287	Presumed	Enzyme induction
	Increased relative thyroid weight / increased circulating TSH level	Metribuzin	<5	5	Unknown	
	Inflammatory and degenerative changes in the thyroid	Pymetrozine	3	14	Unknown	

H. LIST OF EXAMINED SUBSTANCES IN THE DATA COLLECTION (STEP 3) AND THOSE SELECTED FOR CAGS (STEP 4)

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
1-Methylcyclopropene	-	-	-	-
1-Naphthylacetamide (1-NAD)	-	-	1-NAD	-
1-Naphthylacetic acid (1-NAA)	-	-	1-NAA	-
2,4-D	2,4-D	2,4-D	2,4-D	2,4-D
2,4-DB (metabolized to 2,4-D)	-	-	2,4-DB	2,4-DB
2-Phenylphenol (including sodium salt orthophenyl phenol)	-	-	-	-
6-Benzyladenine	-	-	-	-
Abamectin (aka avermectin)	Abamectin	Abamectin	-	-
Acetamiprid	Acetamiprid	Acetamiprid	-	-
Acibenzolar-S-methyl (benzothiadiazole)	-	-	-	-
Aclonifen	-	-	Aclonifen	Aclonifen
Alpha-Cypermethrin (aka alphamethrin)	alpha-Cypermethrin	alpha-Cypermethrin	-	-
Aluminium phosphide	Aluminium phosphide	-	-	-
Aluminium ammonium sulphate	-	-	-	-
Amidosulfuron	-	-	Amidosulfuron	Amidosulfuron
Amitrole (aminotriazole)	-	-	Amitrole	Amitrole
Azimsulfuron	-	-	-	-
Azoxystrobin	-	-	-	-
Beflubutamid	-	-	Beflubutamid	Beflubutamid
Benalaxyl	-	-	Benalaxyl	Benalaxyl
Benfluralin	Benfluralin	-	Benfluralin	Benfluralin
Bensulfuron	-	-	-	-
Bentazone	-	-	-	-
Benthiavalicarb	Benthiavalicarb	-	Benthiavalicarb	Benthiavalicarb
Benzoic acid	Benzoic acid	-	-	-
Beta-Cyfluthrin	Beta-Cyfluthrin	Beta-Cyfluthrin	-	-
Bifenazate	Bifenazate	-	-	-

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
Bifenox	-	-	-	-
Bispyribac	-	-	-	-
Boscalid	-	-	Boscalid	Boscalid
Bromadiolone	-	-	-	-
Bromoxynil	-	-	Bromoxynil	Bromoxynil
Bromuconazole	-	-	Bromuconazole	Bromuconazole
Bupirimate	-	-	Bupirimate	Bupirimate
Buprofezin	-	-	Buprofezin	Buprofezin
Calcium phosphide	-	-	-	-
Captan	-	-	-	-
Carbendazim	-	-	-	-
Carbetamide	Carbetamide	Carbetamide	Carbetamide	Carbetamide
Carboxin	-	-	Carboxin	Carboxin
Carfentrazone-ethyl	-	-	-	-
Carvone	-	-	-	-
Chloridazon (aka pyrazone)	-	-	-	-
Chlormequat (chloride)	Chlormequat	Chlormequat	-	-
Chlorothalonil	-	-	-	-
Chlorotoluron	-	-	-	-
Chlorpropham	Chlorpropham	Chlorpropham	Chlorpropham	Chlorpropham
Chlorpyrifos	Chlorpyrifos	Chlorpyrifos	-	-
Chlorpyrifos-methyl	Chlorpyrifos-methyl	Chlorpyrifos-methyl	-	-
Chlorsulfuron	-	-	-	-
Cinidon ethyl	-	-	-	-
Clethodim	-	-	Clethodim	Clethodim
Clodinafop	-	-	Clodinafop	Clodinafop
Clofentezine	-	-	Clofentezine	Clofentezine
Clomazone	-	-	-	-
Clopyralid	-	-	-	-
Clothianidin	Clothianidin	Clothianidin	-	-
Copper compounds	Copper compounds	-	-	-
Cyazofamid	-	-	-	-
Cyclanilide	-	-	-	-
Cycloxydim	-	-	Cycloxydim	Cycloxydim
Cyflufenamid	-	-	Cyflufenamid	Cyflufenamid
Cyfluthrin	Cyfluthrin	Cyfluthrin	-	-
Cyhalofop-butyl	-	-	Cyhalofop-butyl	Cyhalofop-butyl
Cymoxanil	Cymoxanil	Cymoxanil	-	-

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
Cypermethrin	Cypermethrin	Cypermethrin	-	-
Cyproconazole	-	-	Cyproconazole	Cyproconazole
Cyprodinil	-	-	Cyprodinil	Cyprodinil
Cyromazine	Cyromazine	-	-	-
Daminozide	-	-	-	-
Dazomet	-	-	Dazomet	Dazomet
Deltamethrin	Deltamethrin	Deltamethrin	-	-
Desmedipham	Desmedipham	Desmedipham	Desmedipham	Desmedipham
Dicamba	Dicamba	Dicamba	-	-
Dichlorprop-P	-	-	Dichlorprop-P	Dichlorprop-P
Diclofop	-	-	Diclofop	Diclofop
Diethofencarb	-	-	Diethofencarb	Diethofencarb
Difenoconazole	Difenoconazole	-	-	-
Diflubenzuron	Diflubenzuron	-	-	-
Diflufenican	-	-	-	-
Dimethachlor	-	-	-	-
Dimethenamid-P	-	-	-	-
Dimethoate	Dimethoate	Dimethoate	-	-
Dimethomorph	-	-	-	-
Dimoxystrobin	-	-	-	-
Dinocap	-	-	Dinocap	Dinocap
Diquat (dibromide)	-	-	-	-
Dithianon	-	-	Dithianon	Dithianon
Diuron	-	-	-	-
Dodemorph	-	-	-	-
Dodine	-	-	Dodine	Dodine
Epoxiconazole	-	-	-	-
Esfenvalerate	Esfenvalerate	Esfenvalerate	-	-
Ethephon	Ethephon	Ethephon	-	-
Ethofumesate	-	-	-	-
Ethoprophos	Ethoprophos	Ethoprophos	-	-
Ethoxysulfuron	-	-	-	-
Etofenprox	Etofenprox	-	Etofenprox	Etofenprox
Etoxazole	Etoxazole	-	-	-
Etridiazole	-	-	Etridiazole	Etridiazole
Famoxadone	-	-	-	-
Fenamidone	-	-	Fenamidone	Fenamidone
Fenamiphos (aka phenamiphos)	Fenamiphos	Fenamiphos	-	-
Fenazaquin	-	-	Fenazaquin	-
Fenbuconazole	-	-	Fenbuconazole	Fenbuconazole

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
Fenbutatin oxide	-	-	-	-
Fenhexamid	-	-	-	-
Fenoxaprop-P	-	-	-	-
Fenoxycarb	-	-	Fenoxycarb	Fenoxycarb
Fenpropidin	Fenpropidin	Fenpropidin	-	-
Fenpropimorph	Fenpropimorph	Fenpropimorph	-	-
Fenpyroximate	Fenpyroximate	-	-	-
Fipronil	Fipronil	Fipronil	Fipronil	Fipronil
Flazasulfuron	-	-	Flazasulfuron	-
Flonicamid (IKI-220)	-	-	Flonicamid	-
Florasulam	-	-	-	-
Fluazifop-P	-	-	-	-
Fluazinam	-	-	-	-
Fludioxonil	-	-	-	-
Flufenacet (formerly fluthiamide)	Flufenacet	Flufenacet	Flufenacet	Flufenacet
Flumioxazin	-	-	Flumioxazin	Flumioxazin
Fluometuron	-	-	-	-
Fluopicolide	-	-	Fluopicolide	Fluopicolide
Fluoxastrobin	-	-	Fluoxastrobin	Fluoxastrobin
Flupyrsulfuron-methyl (DPX KE 459)	-	-	-	-
Fluquinconazole	Fluquinconazole	Fluquinconazole	Fluquinconazole	Fluquinconazole
Flurochloridone	-	-	-	-
Fluroxypyr	-	-	-	-
Flurtamone	-	-	-	-
Flusilazole	-	-	-	-
Flutolanil	-	-	Flutolanil	Flutolanil
Flutriafol	-	-	Flutriafol	-
Folpet	-	-	Folpet	Folpet
Foramsulfuron	-	-	-	-
Forchlorfenuron	-	-	-	-
Formetanate	Formetanate	Formetanate	Formetanate	Formetanate
Fosetyl	-	-	-	-
Fosthiazate	Fosthiazate	Fosthiazate	-	-
Fuberidazole	-	-	Fuberidazole	Fuberidazole
Gibberellin	-	-	-	-
Glufosinate	Glufosinate	Glufosinate	-	-
Glyphosate (including trimesium aka sulfosate)	-	-	-	-

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
Haloxyfop-P/R	-	-	Haloxyfop-P/R	Haloxyfop-P/R
Hexythiazox	-	-	Hexythiazox	Hexythiazox
Hymexazol	-	-	Hymexazol	Hymexazol
Imazalil (aka enilconazole)	-	-	-	-
Imazamox	-	-	-	-
Imazaquin	-	-	-	-
Imazosulfuron	-	-	Imazosulfuron	Imazosulfuron
Imidacloprid	Imidacloprid	Imidacloprid	Imidacloprid	Imidacloprid
Indoxacarb	Indoxacarb	Indoxacarb	-	-
Iodosulfuron	-	-	-	-
Ioxynil	-	-	Ioxynil	Ioxynil
Iprodione	-	-	-	-
Iprovalicarb	Iprovalicarb	-	-	-
Isoproturon	-	-	-	-
Isoxaben	-	-	Isoxaben	-
Isoxaflutole	Isoxaflutole	Isoxaflutole	Isoxaflutole	Isoxaflutole
Kresoxim-methyl	-	-	-	-
Lambda-Cyhalothrin	Lambda-Cyhalothrin	Lambda-Cyhalothrin	-	-
Lenacil	-	-	Lenacil	Lenacil
Linuron	-	-	-	-
Lufenuron	Lufenuron	Lufenuron	Lufenuron	Lufenuron
Magnesium phosphide	Magnesium phosphide	-	-	-
Malathion	Malathion	Malathion	Malathion	-
Maleic hydrazide	-	-	Maleic hydrazide	Maleic hydrazide
Mancozeb	Mancozeb	Mancozeb	Mancozeb	Mancozeb
Maneb	Maneb	Maneb	Maneb	Maneb
MCPA	-	-	MCPA	MCPA
MCPB	-	-	-	-
Mecoprop	-	-	-	-
Mecoprop-P	-	-	-	-
Mepanipyrim	-	-	Mepanipyrim	Mepanipyrim
Mepiquat	Mepiquat	Mepiquat	-	-
Mesosulfuron	-	-	-	-
Mesotrione	-	-	-	-
Metalaxyl-M	-	-	-	-
Metaldehyde	Metaldehyde	Metaldehyde	-	-
Metamitron	Metamitron	-	-	-
Metazachlor	-	-	-	-

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
Metconazole	-	-	Metconazole	-
Methiocarb (aka mercaptodimethur)	Methiocarb	Methiocarb	-	-
Methomyl	Methomyl	Methomyl	-	-
Methoxyfenozide	-	-	-	-
Metiram	Metiram	Metiram	Metiram	Metiram
Metosulam	-	-	-	-
Metrafenone	-	-	-	-
Metribuzin	Metribuzin	-	Metribuzin	Metribuzin
Metsulfuron-methyl	-	-	-	-
Milbemectin	Milbemectin	Milbemectin	-	-
Molinate	Molinate	Molinate	-	-
Myclobutanil	-	-	Myclobutanil	Myclobutanil
Napropamide	-	-	-	-
Nicosulfuron	-	-	-	-
Oryzalin	-	-	Oryzalin	Oryzalin
Oxadiargyl	-	-	Oxadiargyl	Oxadiargyl
Oxadiazon	-	-	Oxadiazon	Oxadiazon
Oxamyl	Oxamyl	Oxamyl	-	-
Oxasulfuron	Oxasulfuron	Oxasulfuron	-	-
Oxyfluorfen	-	-	Oxyfluorfen	Oxyfluorfen
Paclobutrazol	-	-	-	-
Penconazole	-	-	-	-
Pencycuron	-	-	-	-
Pendimethalin	-	-	Pendimethalin	Pendimethalin
Penoxsulam	-	-	Penoxsulam	Penoxsulam
Pethoxamid	-	-	Pethoxamid	Pethoxamid
Phenmedipham	-	-	-	-
Phosmet	Phosmet	Phosmet	-	-
Picloram	-	-	Picloram	
Picolinafen	-	-	Picolinafen	Picolinafen
Picoxystrobin	-	-	-	-
Pirimicarb	Pirimicarb	Pirimicarb	-	-
Pirimiphos-methyl	Pirimiphos-methyl	Pirimiphos-methyl	-	-
Prochloraz	-	-	Prochloraz	Prochloraz
Profoxydim (aka Clefoxydim)	-	-	-	-
Prohexadione (including Prohexadione-calcium)	-	-	-	-

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
Propamocarb	Propamocarb		-	-
Propaquizafop	-	-	Propaquizafop	Propaquizafop
Propiconazole	-	-	-	-
Propineb	Propineb	Propineb	Propineb	Propineb
Propoxycarbazone	-	-	-	-
Propyzamide	-	-	Propyzamide	Propyzamide
Proquinazid	-	-	Proquinazid	Proquinazid
Prosulfocarb	-	-	-	-
Prosulfuron	-	-	-	-
Prothioconazole	-	-	Prothioconazole	Prothioconazole
Pymetrozine	Pymetrozine	-	Pymetrozine	Pymetrozine
Pyraclostrobin	-	-	-	-
Pyraflufen-ethyl	-	-	-	-
Pyrethrins	Pyrethrins	Pyrethrins	Pyrethrins	Pyrethrins
Pyridaben	-	-	-	-
Pyridate	Pyridate	Pyridate	Pyridate	Pyridate
Pyrimethanil	-	-	Pyrimethanil	Pyrimethanil
Pyriproxyfen	Pyriproxyfen	-	-	-
Quinmerac	-	-	Quinmerac	
Quinoclamine	Quinoclamine	Quinoclamine	Quinoclamine	Quinoclamine
Quinoxyfen	-	-	-	-
Quizalofop-P (including ethyl and tefuryl)	-	-	Quizalofop-P	Quizalofop-P
Rimsulfuron (aka renriduron)	-	-	-	-
Silthiofam	-	-	Silthiofam	Silthiofam
Sintofen (aka Cintofen)	-	-	-	-
S-Metolachlor	-	-	-	-
Sodium 5-nitroguaiacolate	-	-	-	-
Sodium hypochlorite	-	-	-	-
Sodium o-nitrophenolate	-	-	-	-
Sodium p-nitrophenolate	-	-	-	-
Spinosad	Spinosad	Spinosad	Spinosad	Spinosad
Spirodiclofen	-	-	Spirodiclofen	Spirodiclofen
Spiroxamine	-	-	-	-
Sulcotrione	Sulcotrione		-	-
Sulfosulfuron	-	-	Sulfosulfuron	-
Sulfuryl fluoride	Sulfuryl fluoride	Sulfuryl fluoride	-	-

Substances	Nervous system Step 3	Nervous system Step 4	Thyroid system Step 3	Thyroid system Step 4
tau-Fluvalinate	tau-Fluvalinate	tau-Fluvalinate	-	-
Tebuconazole	Tebuconazole	Tebuconazole	-	-
Tebufenozide	-	-	-	-
Tebufenpyrad	Tebufenpyrad	-	-	-
Teflubenzuron	-	-	-	-
Tefluthrin	Tefluthrin	Tefluthrin	-	-
Tepraloxydim	-	-	Tepraloxydim	Tepraloxydim
Terbuthylazine	-	-	Terbuthylazine	Terbuthylazine
Tetraconazole	Tetraconazole	Tetraconazole	Tetraconazole	Tetraconazole
Thiabendazole	-	-	Thiabendazole	Thiabendazole
Thiacloprid	Thiacloprid	Thiacloprid	Thiacloprid	Thiacloprid
Thiamethoxam	Thiamethoxam	Thiamethoxam	Thiamethoxam	Thiamethoxam
Thifensulfuron-methyl	-	-	-	-
Thiophanate-methyl	Thiophanate-methyl	-	Thiophanate-methyl	Thiophanate-methyl
Thiram	Thiram	Thiram	Thiram	Thiram
Tolclofos-methyl	Tolclofos-methyl	-	-	-
Tolylfluanid	Tolylfluanid	-	Tolylfluanid	Tolylfluanid
Tralkoxydim	-	-	-	-
Triadimenol	Triadimenol	Triadimenol	-	-
Tri-allate	Tri-allate	Tri-allate	-	-
Triasulfuron	-	-	-	-
Triazoxide	-	-	-	-
Tribenuron (aka metometuron)	-	-	Tribenuron	Tribenuron
Triclopyr	-	-	-	-
Trifloxystrobin	-	-	-	-
Triflumizole	-	-	-	-
Triflumuron	-	-	-	-
Triflusulfuron	-	-	-	-
Trinexapac (aka cimetacarb ethyl)	-	-	-	-
Triticonazole	-	-	-	-
Tritosulfuron	-	-	Tritosulfuron	Tritosulfuron
zeta-Cypermethrin	zeta-Cypermethrin	zeta-Cypermethrin	-	-
Zinc phosphide	-	-	-	-
Ziram (including impurity TMTU)	Ziram	Ziram	Ziram	Ziram
Zoxamide	-	-	Zoxamide	Zoxamide
Total = 287	**Total = 91**	**Total = 67**	**Total = 113**	**Total = 101**

Notes

1. Substances considered not exerting relevant effects and thereby not included in the data collection are marked with the symbol –
2. Substances that are not highlighted in any colour are those approved until 31st of May 2009 and evaluated by both DTU, RIVM and EFSA
3. Substances highlighted in green are those approved between 31st of May 2009 and 1st of January 2012, and evaluated by RIVM and EFSA
4. Substances highlighted in red are those approved prior to 31st of May 2009 but not evaluated by DTU and therefore added to the list and evaluated by RIVM and EFSA