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Helsinki, 13 February 2018

Substance name: Ziram
EC number: 205-288-3
CAS number: 137-30-4
Date of Latest submission(s) considered[1]: 12 March 2015
Decision/annotation number: Please refer to the REACH- IT message which delivered this communication (in format SEV-D-XXXXXXXXXX-XX-XX/F)
Addressees: Registrant(s)[2] of Ziram (Registrant(s))

DECISION ON SUBSTANCE EVALUATION

1. Requested information

Based on Article 46(1) of Regulation (EC) No 1907/2006 (the 'REACH Regulation'), you are requested to submit the following information on the registered substance:

- **Combined Developmental Neurotoxicity study (OECD TG 426) and Neurotoxicity study in rats (OECD TG 424), oral route of administration via feed, including additional investigations in the OECD TG 424 part of the study as specified in Appendix 3.**

  The Developmental Neurotoxicity Study in rodents (OECD TG 426) shall be conducted according to the OECD test guideline. The adult animals from this study (F0) shall be kept and tested according to a Neurotoxicity study in rodents (OECD TG 424) with additional investigations as further specified in Appendix 3 of this decision: The number of investigated animals shall be sufficiently high, the dose levels shall be wide-ranged and a range-finding study may be necessary, the dosing period shall be at least 90 days for the OECD TG 424 part of the study in addition to the period for the OECD TG 426 part of the study, a number of specified functional tests at the right time shall be included, and specified histopathological methods and investigations shall be included in addition to the mandatory histopathological investigation presented in the guideline.

You have to provide an update of the registration dossier(s) containing the requested information, including robust study summaries and, where relevant, an update of the Chemical Safety Report by **20 November 2020**. The deadline takes into account the time that you may need to agree which of the registrant(s) will perform the required tests.

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[1] This decision is based on the registration dossier(s) on the day until which the evaluating MSCA granted an extension for submitting dossier updates which it would take into consideration.

[2] The terms Registrant(s), dossier(s) or registration(s) are used throughout the decision, irrespective of the number of registrants addressed by the decision.

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The evaluating Member State Competent Authority (MSCA) must have access to the full study report including all relevant details of the study, ensuring that a clear conclusion regarding the result of the study can be drawn by the evaluating MSCA.

The reasons of this decision are set out in Appendix 1. The procedural history is described in Appendix 2. Further information, observations and technical guidance as appropriate are provided in Appendix 3. Appendix 4 contains a list of registration numbers for the addressees of this decision. This appendix is confidential and not included in the public version of this decision.

2. Who performs the testing

Based on Article 53 of the REACH Regulation, you are requested to inform ECHA who will carry out the study/ies on behalf of all Registrant(s) within 90 days. Instructions on how to do this are provided in Appendix 3.

3. Appeal

This decision can be appealed to the Board of Appeal of ECHA within three months of its notification. An appeal, together with the grounds thereof, shall be submitted to ECHA in writing. An appeal has suspensive effect and is subject to a fee. Further details are described under http://echa.europa.eu/regulations/appeals.

Authorised[3] by Leena Ylä-Mononen, Director of Evaluation

[3] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

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Appendix 1: Reasons

Based on the evaluation of all relevant information submitted on Ziram and other relevant available information, ECHA concludes that further information is required in order to enable the evaluating Member State Competent Authority (MSCA) to complete the evaluation of whether the substance constitutes a risk to human health and the environment.

The evaluating MSCA will review the information submitted by you and evaluate if further information should be requested in order to clarify the concerns for developmental neurotoxicity and/or parkinsonian disorders.

Ziram is registered under REACH at a tonnage band of 100-1000 tonnes per annum. The registrations cover the manufacture, formulation and industrial use as a vulcanisation agent in rubber and latex production in the EU. Thus, there is potential for exposure of workers and the environment.

It is noted that Ziram is approved for use as an active pesticide substance in the EU according to EC Regulation 1107/2009, and is currently under re-evaluation. The evaluating MSCA for substance evaluation under Regulation (EC) No 1907/2006 (the 'REACH Regulation') has obtained information from this pesticide draft renewal assessment report on Ziram from EFSA (draft RAR).

The draft RAR concludes that developmental neurotoxicity is observed as effects on behaviour of offspring at specific timepoints in one study ([redacted]), and a NOAEL is set based on these observations. Since also some systemic toxicity is observed in the dams and offspring at the same dose level, it is, however, concluded that Ziram is not a specific developmental neurotoxicant. ECHA finds that developmental neurotoxicity of Ziram is not clarified since the study was not in accordance with the guideline as several endpoints for developmental neurotoxicity (startle response, cognitive function) were not assessed. These drawbacks in the study design are not discussed in the draft RAR.

Furthermore, the draft RAR does not include the studies which raise a concern that exposure to the registered substance can lead to parkinsonian disorders (Lulla et al., 2016, Wang et al., 2011, Fitzmaurice et al., 2014, Chou et al., 2008, Wang et al., 2006.).

It should be noted that the draft RAR was withdrawn in June 2017 since the nature of the comments received indicated that substantive changes were required, and this was considered unlikely to be possible to complete within the short timeframe of the process.

Concern for developmental neurotoxicity and parkinsonian disorders

The Concerns Identified

The data available on Ziram raise two concerns, but do not lead to clear conclusions regarding these two concerns: Developmental neurotoxicity and parkinsonian disorders.

Concern for developmental neurotoxicity

Two studies have been performed indicating effects of Ziram on the developing nervous system in rats. A developmental neurotoxicity study in rats (design based on OECD TG 426) showed significant increases in motor activity levels in young and adult offspring ([redacted]), whereas a 2-generation reproductive toxicity study with behavioural

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testing of F2 offspring showed non-significant increases in motor activity levels ([redacted]). Based on these two studies, a concern is raised, but no final conclusions can be drawn. Both studies are described below.

Developmental neurotoxicity study in rats [redacted] performed a dietary developmental neurotoxicity study in rats using a test design based on OECD Guideline for Testing of Chemicals, Guideline 426, Developmental Neurotoxicity Study, 16 October 2007 (according to GLP). However, the study was not in accordance with the guideline as several endpoints for developmental neurotoxicity (startle response, cognitive function) were not assessed. Purity of Ziram was 96-75%. Mated female (species:Crl:CD(SD), Charles river laboratories (Sprague Dawley)) rats were exposed to doses of 0, 72, 207, 540/360 ppm in diet from gestation day 6 to lactation day 21. The high dose diet concentration was reduced from 540 ppm to 360 ppm on lactation day 4 due to maternal toxicity at the highest dose. Calculated test substance consumptions were 5, 13 and 27 mg/kg bw/day during gestation; 8, 23 and 59 mg/kg bw/day during lactation days 1 to 4, and 12, 34 and 61 mg/kg bw/day during lactation days 4 to 21.

The study included 25 mated females per exposure group. 20 pups per sex per group were selected for motor activity evaluations and post-weaning developmental landmarks. Brain weights and brain morphometrics were analyzed in a subset of these animals (15 and 10 per sex per group, respectively) on post-natal day (PND) 72. A second subset of 15 pups/sex/group was selected for brain weight evaluations on PND 21; of these, 10 pups/sex from the control and high-exposure groups and 10 male pups from the low- and mid-exposure groups were selected for neuropathological and morphometric evaluations on PND 21.

Maternal body weights were decreased by 15% at the high dose of 360/540 ppm compared to controls at gestation day (GD) 20; and by 15% at PND 1. This was a marked reduction in maternal body weight and can be considered as maternal toxicity at the high dose level. At the middle dose of 207 ppm maternal body weights were decreased by 3.8% compared to controls at GD 20 and by 3% at PND 1. In that dose group, maternal body weight gain was reduced mainly at GD 18 to 20, and the maternal effect of Ziram can be considered minor. Pup weights were decreased by 11% in the 360/540 ppm group compared to controls at PND 1 and the lower pup weights continued throughout lactation. In the group exposed to 207 ppm pup weights were not decreased in the early postnatal period but were decreased in male pups PND 21 (8.4%) and not in females PND 21 (6.8%, not statistically significant).

Locomotor activity was assessed for 20 rats/sex/group on PND 13, 17, 21 and 61. Mean overall locomotor activity (total and ambulatory counts) was increased in the 207 ppm group males and females on PND 21 and in the 540/360 ppm group on PND 13, 17 and 21. The increases in mean overall locomotor activity counts correlated with less habituation to the test environment on PND 17 and/or 21 in the 207 and 540/360 ppm groups, may at 540/360 ppm but not 207 ppm be related, in part, to developmental delay. Significantly increased mean locomotor counts were also found in the 207 and 540/360 ppm group males and females on PND 61, both when the sexes were analyzed together and when separated by sex. In the study report, the effect in the 207 and 540/360 ppm groups on PND 61 were not considered exposure-related and the author's arguments for this were: "generally small magnitudes of change during the individual subintervals, absence of a clear exposure-related trend and the lack of any remarkable effect on the pattern of habituation". Increased activity may, however, be an overall effect not related to an effect on habituation or to changes during specific subintervals.

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Also, the absence of an exposure-related trend (i.e. higher activity in the 540/360 ppm group than the 207 ppm group) may be due to the relatively small difference in dose between these two groups. Therefore, ECHA does not consider these arguments sufficient in order to dismiss the significantly increased activity found in both males and females at the two highest dose levels.

Mean locomotor activity counts in the 72 ppm group were unaffected by maternal test diet exposure at all testing ages. Post-weaning developmental landmarks (age of balanopreputial separation and vaginal patency) for the F1- males and F1-females were also unaffected by the maternal test diet exposure. Weight at balanopreputial separation was, however, significantly reduced in males from the high dose group.

In males, reduced brain weight, reduced brain size and decreased radial thickness of the cerebral cortex were observed in the 270 and 540/360 dose groups at PND21. This may partly be due to lower body weight in these dose groups. In females, reduced weight and size of the brain were observed in the high dose on PND 72. In this group, the mean body weight was similar to control females. Relative brain weights were not significantly affected in either males or females on day 21 or on day 72 ([redacted]).

2-generation reproductive toxicity [redacted])
In a 2-generation reproductive toxicity study on Ziram ([redacted]), behavioral testing of F2 offspring was also performed. The study protocol was in compliance with test method B.5 in Commission Directive 87/302/EEC of 18 November 1987. The study was performed in Sprague Dawley (SD) rats (30 /sex/dose) exposed via feed to 0, 60, 180, 540 ppm of Ziram (Concentration was increased to 72 ppm (instead of 60 ppm)/ 207 ppm (instead of 180 ppm) to compensate losses during storage). Exposure: F0 parents: from study initiation until scheduled sacrifice. F1 parents: from weaning (day 22) until scheduled sacrifice. F2 pups: from weaning (day 22) until scheduled sacrifice. Duration of exposure before mating (F0 / F1 parents): 10 weeks (Ad libitum).

Reduced body weight and food consumption were seen in F0 and F1 dams in doses from 60 ppm, and decrease in mean pup body weight in the F1 generation was seen in the high dose group. Reproductive parameters such as fertility, mating, days between pairing and coitus, gestation and parturition were not adversely affected by Ziram administration.

Motor activity test, Auditory startle test, and Biel Maze swimming trials were performed in F2 offspring (10 rats per sex per dose) and did not reveal any significant differences between controls and Ziram exposed groups. However, a tendency towards increased motor activity in Ziram exposed offspring was seen in this study. This study applied only 10 animals per sex per dose group for the activity test (while current OECD test guidelines for developmental neurotoxicity testing recommend to use 20 animals per sex per dose group), and although the observed tendency was not statistically significant, this finding corroborates the statistically significant results by [redacted]. In this smaller study, reduced brain weights were observed in high dose F1 males and relative, but not absolute, brain weights were increased in F0 and F1 females. These findings may be attributed to body weight reductions. In F2 offspring no effects were seen in a neuropathological/histomorphological examination at PND 11, and the only change observed was an 8% increase in relative brain weight of high dose males.

Conclusion on developmental neurotoxicity
Together, the studies by [redacted] and [redacted] indicate that Ziram may cause adverse effects on the developing nervous system manifested as increased activity

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before weaning and in adulthood, but no final conclusions can be drawn based on these studies.

The concern for developmental neurotoxicity does not exclude the concern for parkinsonian disorders, which are further described below. Ziram may act through more than one mode of action leading to several effects during the development and lifetime of an organism.

In this case, there is a concern for developmental neurotoxicity due to the increased motor activity observed in the offspring after exposure in utero (in [redacted] and non-significantly in [redacted]), and there is a concern for parkinsonian disorders, as described below.

Concern for parkinsonian disorders

The term parkinsonian disorders is used here as defined in the scientific opinion of the PPPR Panel (EFSA 2017): "*Parkinson's disease is a chronic progressive neurodegenerative disorder with a higher prevalence in the aged male population... Although the clinical symptoms include slowness of movement, resting tremor, rigidity and disturbances in balance, it is recognized that additional non-motor symptoms can occur as a result of the progression of the disease. Some or all of the motor symptoms can, however, be observed in different disorders and the resulting syndrome is called 'parkinsonism'. When parkinsonism is the prominent part of the disorder, these are referred to as 'parkinsonian disorders' and include Parkinson's disease. The primary pathology is, however, common to all parkinsonian disorders and is represented by a selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which project mainly to the striatum, in association with the development of cytoplasmic, protein-rich inclusions, called Lewy-bodies (LB) and decreased levels of striatal dopamine. One of the main components of LB is the aberrant oligomeric α-synuclein (a presynaptic neuronal protein) and a parallelism exist between the presence of motor and non-motor symptoms and the finding of α-synuclein pathology beyond the SNpc*".

There is a concern that exposure to the registered substance can lead to parkinsonian disorders. This concern is based on epidemiological and mechanistic studies. Epidemiological studies (Wang et al., 2011 and Fitzmaurice et al., 2014) indicate that exposure to Ziram is associated with development of Parkinson's Disease, which is a common neurodegenerative disease characterized by a relatively selective degeneration of dopaminergic neurons in the substantia nigra (nigrostriatal neurons) (Chou et al., 2008). This suggested association is further supported by mechanistic studies lending support and biological plausibility to the epidemiological data (as outlined below).

Epidemiological studies

Epidemiological studies suggest that exposure to some pesticides, including Ziram, is associated with increased relative risk for developing Parkinson's Disease (Wang et al., 2011 and Fitzmaurice et al., 2014). In Wang et al. 2011, ambient exposures to Ziram, maneb and paraquat at work places and residences in California's central valley from 1974 to 1999 were estimated by use of a pesticide use reporting system. Exposure to Ziram (N=341 for cases and controls) was associated with an Odds Ratio (OR) of 1.13, 1.52 and 3.01 for developing Parkinson's Disease if exposed at residences only, workplaces only and in both locations, respectively. Co-exposure to maneb and/or paraquat could not be excluded for these cases. However, when looking at cases with only Ziram exposure (not maneb or paraquat exposure), the ORs were also above 1, even though the number of participants were much smaller. The ORs were 1.48 and 1.37

for developing Parkinson's Disease if exposed at residences (N=4 for cases, N=3 for controls) or workplaces (N=6 for cases and controls), respectively (Wang et al., 2011). In Fitzmaurice et al. (2014), exposures to Ziram and a number of other pesticides were estimated based on the same pesticide use reporting system for the same geographical location and period of time, but with a modified approach for OR estimation. Exposure to Ziram was associated with an OR of 0.77, 1.71 and 3.33 for developing Parkinson's Disease if exposed at residences only, workplaces only and in both locations, respectively (Fitzmaurice et al., 2014).

There are obvious limitations to these two studies, as well as to many other epidemiological studies investigating associations between exposures and effects in humans. These drawbacks include the low number of cases included in the studies, the lack of an accurate exposure estimate due to the long latency period of parkinsonian disorders and that people often have been exposed to more than one pesticide during this period. Furthermore, lifestyle, environmental and genetic risk factors may exist, which may not be sufficiently accounted for in such epidemiological studies (EFSA 2017).

In the scientific opinion from the EFSA PPPR Panel from 2017, it is concluded that the overall appraisal of meta-analyses available so far suggests that there is sufficient evidence to conclude on an association between pesticide exposure (broad definition) and parkinsonian disorders, but not enough to support a causal relationship with specific pesticides classes or compounds (EFSA 2017).

According to the scientific opinion from the EFSA PPPR Panel from 2017, the ultimate goal is that experimental and mechanistic data lend support and biological plausibility to the human epidemiological data. Further, it is highlighted that the concept of AOP (Adverse Outcome Pathways) can help in supporting biological plausibility by means of linking a molecular initiating event to an adverse outcome which is relevant for a given disease. The scientific opinion also highlight that complex and multihits diseases, like parkinsonian disorders, will benefit of this approach by identifying molecular initiating event(s) triggering the adverse outcome, thus identifying potential risk factors. For Ziram, mechanistic data are available which fits into the AOPs described in the scientific opinion from the EFSA PPPR Panel, and thereby lend support and biological plausibility to the human epidemiological data.

AOP considerations and mechanistic studies
In the scientific opinion from the EFSA PPPR Panel, two AOPs (Adverse Outcome Pathways) relevant for parkinsonian disorders are suggested. It is acknowledged that the AOP concept has not been developed to cover complex human diseases and that many different AOPs may be associated to the disease, since diseases may follow a multihit model instead of linear chains of events and may have multiple symptoms instead of one final health outcome. The suggested AOPs are thus model AOPs to pave the way for further development within the area. One of the AOPs suggested by the EFSA PPPR Panel has in the spring 2017 been through the last review round in the OECD AOP Wiki programme, and is expected to be added to the OECD AOP Wiki in 2017. The second AOP is suggested, but not yet further reviewed within the OECD.

Some key events are common in the two suggested AOPs, i.e. the degeneration of dopaminergic neurons of the nigrostriatal pathway is a key event on organ level, and impaired proteostasis is the cellular key event leading to this degeneration of neurons (EFSA 2017).

Ziram is shown in mechanistic laboratory studies (Chou et al., 2008, Lulla et al., 2016

and Wang et al., 2006) to lead to both impaired proteostasis and degeneration of dopaminergic neurons, though the molecular initiating event of Ziram seems to differ from the AOPs described in details in the scientific opinion from the EFSA PPPR Panel. As outlined below, both aldehyde dehydrogenase (ALDH) inhibition and ubiquitin protease system (UPS) inhibition are proposed as a first step in a sequence of events leading to impaired proteostasis and degeneration of dopaminergic neurons after exposure to Ziram.

Ziram has been shown to cause selective dopaminergic cell-loss, measured as a decrease in tyrosine hydroxylase (TH)-positive cells in rat primary mesencephalic cell cultures *in vitro* at 0.5 and 1µM (Chou et al., 2008).

Also in zebra fish embryos, which are used as a model for development of parkinsonian disorders, exposure to Ziram has been shown to cause selective dopaminergic neuron damage and decreased thyrosine hydroxylase protein levels at 50nM (Lulla et al., 2016). Further, the dopaminergic neuron damage was shown to be synuclein-dependent, and to lead to changes in swimming behavior 7 days post fertilization at 50nM (Lulla et al., 2016).

One sign of impaired proteostasis is increased expression and/or aggregation of α-synuclein, which has been shown to increase the risk of developing Parkinson's Disease (Lulla et al., 2016). Ziram has been shown to increase α-synuclein expression in TH-positive cells in rat primary mesencephalic cell cultures in vitro at 1µM (Chou et al., 2008).

Chou et al. (2008) and Wang et al. (2006) suggest that Ziram acts through inhibition of the ubiquitin protease system (UPS) (by inhibition of ubiquitin E1 ligase) as a first step in a pathway leading to impaired proteostasis, protein aggregation and dopaminergic cell death (Chou et al., 2008 and Wang et al.,2006).

The UPS works through the attachment of multiple ubiquitin molecules to a protein substrate, followed by the subsequent degradation of the tagged protein by the proteasome. A compromised function of the UPS leads to the accumulation of ubiquitinylated proteins, such as α-synuclein (Ii et al., 1997; Spillantini et al., 1997; Sulzer and Zecca 2000, EFSA 2017). The accumulation of polyubiquitinated proteins, as a consequence of a dysfunctional proteasome activity, is observed in some pathologies, and experimental inhibition of the proteasome has been shown to trigger parkinsonian neurodegeneration (Hardy et al., 2001; McNaught and Jenner 2001, EFSA 2017).

Ziram has been shown to inhibit the UPS in Sk-N-MC cells (human neuroblastoma cell line) *in vitro* at 1 and 10µM (Wang et al., 2006, Chou et al., 2008) and in HEK cells (human embryonic kidney cell line) with an IC_{50} of 161 nM (Chou et al., 2008). It has also been shown that Ziram reduced UPS E1 ligase activity *in vitro* at 0,5 and 1µM as measured by a reduction of endogenous E1-ubiquitin conjugates and reduced formation of E1-ubiquitin conjugates in a purified preparation (Chou et al., 2008).

Fitzmaurice et al. (2014) suggest that Ziram acts through inhibition of ALDH as a first step in a pathway leading to toxic aldehydes (e.g. DOPAL), protein aggregation and dopaminergic cell death (Fitzmaurice et al., 2014). ALDH enzymes are responsible for detoxification of exogenous and endogenous aldehydes by oxidising aldehydes to carboxylic acids. Aldehyde metabolites have been suggested to be involved in the pathogenesis of parkinsonian disorders; for instance, 4-hydroxy-nonenal (4-HNE), a common aldehyde product of lipid peroxidation, promotes the formation of α-synuclein

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oligomers. ALDH also continuously detoxifies 3,4-dihydroxyphenylacetaldehyde (DOPAL). This degradation product of dopamine is generated in neurons by monoamine oxidase (MAO), and has been involved in the loss of dopaminergic neurons in parkinsonian disorders as a result of generating hydroxyl radicals (EFSA 2017).

Fitzmaurice et al. (2014) show that Ziram inhibits ALDH activity at 10µM in suspension of neurons derived from the substantia nigra of newborn rats (Fitzmaurice et al., 2014). Less emphasis is, however, to be put on this finding, since in Chou et al. (2008) it is reported that Ziram was highly toxic to all cells at 5 and 10 µM *in vitro* (Chou et al., 2008).

Only limited toxicokinetic data are available to inform whether the observations in the mechanistic studies (in vitro and in zebrafish embryos) would be expected to occur in vivo under realistic exposure. In the Ziram pesticide Draft Assessment Report (DAR) (DAR 2004), the following is stated: "Distribution has not been studied in details: quite low levels of radioactivity (1.1-0.01%) were retained 7 days after oral administration and localised in the excretion organs (liver, kidneys, lungs), the carcass and blood. Recovery of radioactivity was < 0.01% in bone, brain, fat, muscle, gonads, spleen, pituitary gland and thyroid gland. After repeated exposure, recovery of radioactivity in tissues and carcass was low 7 days after exposure, suggesting that the compound does not accumulate (Cheng, 1989, task force study)."

However, ECHA evaluates that these data inform about lack of accumulation, but not about lack of distribution to the brain, and that it is not unlikely that sufficient amounts of Ziram may pass through the brain and induce adverse effects.

In vivo studies
Two neurotoxicity studies have been conducted in adult rats, one with acute and one with subchronic exposure to Ziram. Based on information on this in the DAR on Ziram (DAR 2004) summaries of two neurotoxicity studies are presented below.

Acute exposure [redacted]
In a study by [redacted] neurotoxicity was assessed in SD rats (n=12) following gavage administration of a single dose of 15, 300 or 600 mg/kg bw.

Functional Observation Battery (FOB) and motor activity evaluations were performed during the pretest, at the time of peak (approximatively 4 hours post-dosing or day 0) and on day 7 and 14. Sensory and neuromuscular observations were performed during open field tests. At 15 mg/kg, possible treatment related clinical sign of rales were observed on day 1. Furthermore, altered posture and slight impairment of gait were observed during day 0 in this dose group. Impairment of walk, tiptoe gait and impaired mobility were observed at 300 and 600 mg/kg bw, and altered tail pinch and touch was noted at 600 mg/kg bw. At these doses also total activity was reduced, and absolute brain weight was reduced. However, no treatment related microscopic lesions were observed in any of the central or peripheral nervous system tissues examined from the 5 animals/sex in the 600 mg/kg group.

Body temperature was reduced on day 0, 7, and 14 from 300 mg/kg/bw up to 600 mg/kg bw. No description of signs of the general systemic toxicity during treatment and the 14 post-treatment days (i.e. food consumption, body weight etc.) was available in the relatively short study description in the DAR. It was, however, concluded that neuromuscular and CNS activity domains appeared to have been slightly affected at a dose level of 15 mg/kg, but the NOAEL for both neurotoxicity and systemic toxicity was

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set at 15 mg/kg.

90 day exposure ([redacted])
Neurotoxicity assessment has also been performed following 90-day Ziram exposure ([redacted]). In this study, SD rats (n= 10/sex/dose) received oral administration in the diet of 72, 207, 540 ppm (corresponding to 5, 14, 34 mg/kg bw/d for males and 6, 16, 40 mg/kg bw/d for females). In each group, 5 animals/sex/dose were allocated to cholinesterase/neuropathy target esterase (NTE) evaluations, and 5 animals/sex/dose to a neuropathology evaluation.

There were no apparent clinical signs observed in the animals, and no Ziram related effects indicative of neurotoxicity were apparent when Functional Observation Battery (FOB) and Locomotor Activity evaluations were performed.

Brain neuropathy target esterase (NTE) was significantly inhibited at the top dose. No remarkable differences from the control group were observed in mean brain cholinesterase values at any dose of Ziram during study weeks 3, 7, and 13. Absolute and relative brain weights were normal, and no treatment related neuropathological lesions were observed at the microscopic examination of perfused tissues for 5 animals/sex in the 540 ppm group.

The conclusion in the DAR was that repeated dosing of Ziram in the rat did not induce behavioural and neuronal toxicity, and that the toxicological significance of the moderate inhibition of the brain NTE was not clear.

NTE is an integral membrane protein in vertebrate neurons, which has been shown to play an important role in neural development, possibly via involvement in a signaling pathway between neurons and glial cells (Glynn P 2000). NTE was originally discovered as the primary target for those organophosphorus esters (OPs) which cause delayed neuropathy, with degeneration of long axons in peripheral nerves and spinal cord. The size of the reduction in NTE in this study is not presented in the DAR. However, since NTE seems to play an important role in neural development, it is possible that decreased NTE levels may influence the possible developmental neurotoxicity of Ziram.

The evaluating MSCA and ECHA have not had access to the full study reports on the acute and 90 day studies ([redacted]) and the robust study summaries in the Registration dossier were evaluated.

In your comments on the draft decision, you informed that investigation of effects on rotarod performance were included in the 90-days study performed by [redacted]. Since no information about these investigations were included in the robust study summaries in the registration dossier or in your comments, the evaluating MSCA and ECHA have not been able to evaluate these investigations. For example, the power of the test to detect changes is unknown (it cannot be evaluated at which timepoint(s) the investigations were performed or how many animals were included in the investigations). However, even if no effects in these behavioral investigations were observed in the [redacted] study, it would not be sufficient to dismiss the concern for parkinsonian disorders, since the most sensitive and important endpoints for investigation of parkinsonian disorders (i.e. specific sectioning of the brain and staining of special areas with relevant staining methods) were not included in this study.

Since also the [redacted] study did not include the most sensitive endpoints for

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investigation of parkinsonian disorders (i.e. specific sectioning of the brain and staining of special areas with relevant staining methods), access to the full study reports for [redacted] would not change the current decision.

Some functional and/or morphological examinations relevant to the nervous system were included in several other existing toxicity studies with Ziram. No signs of parkinsonian disorders were observed in these studies ([redacted] and Hodge, 1956). As for the studies described above [redacted], none of these studies were designed to investigate specific neurodegenerative effects, and they did not include the most sensitive and important endpoints for investigation of development of parkinsonian disorders (i.e. specific sectioning of the brain and staining of special areas with relevant staining methods).

Parkinsonian motor deficits and degeneration of dopaminergic neurons of the nigrostriatal pathway (which are in common to both AOPs developed by the EFSA PPPR Panel and also suggested to be affected by Ziram based on the mechanistic studies), would most likely be missed in standard regulatory studies, even if serious adverse effects were present (e.g. loss of 30% of all nigral dopaminergic neurons). The most sensitive endpoints for investigation of parkinsonian disorders are specific investigation of relevant brain areas using specific immunohistochemical approaches. Further, clinical signs of parkinsonian disorders in rodent models are best assessed by investigating motor deficits such as coordination, balance and gait abnormalities. None of these endpoints are included in standard regulatory guideline studies (EFSA 2017).

This is further elaborated on in this text from the scientific opinion from the EFSA PPPR Panel (EFSA 2017): "*The AO [Adverse Outcome] (parkinsonian motor deficits) and the KE [Key Event] linked to degeneration of DA [Dopaminergic] neurons of the nigrostriatal pathway (which are common to both AOPs developed by the Panel) are typical apical endpoints that would in theory be identifiable in the regulatory toxicity studies. However, a review of the standard technology and approach used for such studies, showed that changes in these endpoints would most likely be missed, even if large adverse effects were present (e.g. loss of 30% of all nigral dopaminergic neurons). The identification of neuropathology would require specific sectioning of the respective area (which is not done in standard OECD 90-day guideline studies), and it would require immunohistochemical approaches instead of standard H/E staining [standard Haematoxylin and Eosin staining]. The motor deficit would also not be identifiable if neuronal loss in the nigrostriatal pathway was below the threshold activating motor deficits (i.e. below 50–70% loss).*

The lessons learned from the AOP suggest that even if histological sectioning of the substantia nigra and staining for dopaminergic markers were included in a guideline study, severe adverse effects of test chemicals may still be missed. Both AOPs indicate that the perturbation of the key events shows not only a response–response concordance, but also that triggering of some downstream KE requires disturbance of the upstream KE for a prolonged period of time. This has major implications for the study design. For instance, dosing should be tailored in a way to continuously trigger the MIE [Molecular Initiating Event] for a long time. This may not be the case, if toxicants are dosed only once or twice a week, and only three to four times altogether. With an inappropriate dosing schedule, changes in the downstream KE or AO (i.e. the apical endpoints of regulatory studies) may be very low, or even absent. In view of these considerations, AOPs, and the mechanistic information derived from them should be

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used. Optimising the design of hazard identification studies according to the expected mechanisms of toxicity can then be achieved. Moreover, AOP can be used to indicate data gaps in cases of inconsistent experimental studies, and to provide guidance for improved study design to address data gaps, inconsistencies and uncertainties. This also comprises suggestions on additional endpoints to be assessed, either as direct indicators of hazard or as mechanistic support to improve data interpretation and species extrapolation".

Conclusion on concern for parkinsonian disorders
Together, the available mechanistic and epidemiological studies raise a concern that Ziram exposure may induce parkinsonian disorders, but no final conclusions can be drawn based on these studies.

Since no neuropathological investigations specific to assessment of development of parkinsonian disorders were performed in the already available *in vivo* studies, these studies cannot be used to negate this concern.

Why new information is needed
Based on the information described above, there are two concerns: Developmental neurotoxicity and parkinsonian disorders. No final conclusions can be drawn regarding these two concerns based on the available studies.

The concern for developmental neurotoxicity does not exclude the concern for parkinsonian disorders. Ziram may act through more than one mode of action leading to several effects during the development and lifetime of an organism.

What is the possible regulatory outcome
The results of the requested combined Developmental Neurotoxicity study (OECD TG 426) and Neurotoxicity study (OECD TG 424) shall, amongst other relevant and available information, be used to evaluate whether the registered substance should be classified as a reproductive toxicant and/or should be classified STOT RE. From there it will also be assessed whether the substance could be proposed for identification as a substance of very high concern (SVHC) under Article 57(c) or Article 57(f) of REACH, which would lead to stricter risk management measures than those currently in place.

Considerations on the test method and testing strategy
Further specifications for the testing are included in Appendix 3.

You shall submit the full study report for the requested study. Considering the complexity of the study, a complete rationale and access to all information available in the full study report (implemented method, raw data collected, interpretations and calculations, consideration of uncertainties, argumentation, etc.) are needed. This will allow the evaluating MSCA to fully assess the provided information, including the statistical analysis, and to efficiently clarify the concern for developmental neurotoxicity and parkinsonian disorders. The need for access to the full study report is especially warranted in this case, because the requested study includes specific additional investigations of relevance for elucidating key events linked to parkinsonian disorders, investigations that are in addition to those already included in a combined OECD TG 426 and OECD TG 424 study.

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Alternative approaches

The request for a combined OECD TG 426 and OECD TG 424 study is suitable and necessary to obtain information that will allow to clarify the concerns for developmental toxicity and parkinsonian disorders. More explicitly, there is no equally suitable alternative way available of obtaining this information. Currently no *in vitro* or *in silico* alternatives exists that could replace the information obtained in the requested study. ECHA notes that there is no experimental study available at this stage that will generate the necessary information and does not need to test on vertebrate animals.

Endpoints for examination of developmental neurotoxicity are also included in the extended one-generation reproductive toxicity study (OECD 443), and this study could also be an option, but would not be as useful as the requested combined OECD 424/426, as OECD 443 only includes limited testing for developmental neurotoxicity even when the DNT cohort is included.

One of the PfAs disagreed with requesting a study in rats since it is noted that the EFSA review (page 103) raised concerns regarding the validity of the rat as a reliable model for investigation of Parkinson's Disease

It is acknowledged that the rat model may not be the most sensitive model when investigating parkinsonian disorders. It is noted that the rat model for example does not capture all the symptoms of parkinsonian disorders that are observed in humans. However, the AOPs defined in the scientific opinion from the EFSA PPPR Panel (EFSA 2017) are based on parkinsonian motor symptoms which are an adverse outcome that is universally present in human cases of parkinsonian disorders and which has a well-defined underlying pathology that can be induced and captured in animal models (including rats).

An alternative to using the rat model, as suggested by the evaluating MSCA, would be to use genetically modified mice to investigate the concern for development of parkinsonian disorders. This would, however, require the request of an additional animal- and resource-intensive study, which is considered to be disproportionate, especially since the scientific opinion from the EFSA PPPR Panel (EFSA 2017) points out that the drawbacks of the rat as a model should be considered when studies are designed. This is interpreted by ECHA as meaning that the rat model can be used, but that it is important to include the specific investigations, which are included in the OECD TG 424 part of the requested study.

This interpretation is further supported by the recommendations in the scientific opinion from the EFSA PPPR Panel (EFSA 2017) (at page 62-63), which includes the following bullet points:

> "- *The Panel recommends that for compounds affecting the AOPs developed for Parkinsonian motor symptoms, the evaluation of the nigrostriatal pathway should be performed by means of application of proper stereology protocols and detailed neuropathology assessment with inclusion of special stain procedure in addition to H/E to examine whether there is evidence for neuronal cell loss, cell damage and any neuroinflammatory response. It is important that an unbiased assessment of neuronal cell loss must include carefully conducted stereology and pathology studies where the pathologist is 'blinded' to the treatment regimen of each experimental animal assessed. Furthermore, relevant neurochemistry toxicity*

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endpoints should also be examined. The Panel also recommends that biomarkers, e.g. a-synuclein could be considered to help in the study design, i.e. dose selection and length of the treatment, when compounds are known to affect the pathway but the regulatory endpoints are negative.

- The Panel recommends that the standard OECD guidance on histological evaluation of the brain in the 90-day toxicity study (OECD TG 408) and in general in the toxicity studies performed in vivo, should be revised in order to include a more in depth evaluation of brain structures involved in Parkinson disease i.e. the nigrostriatal pathway."

Taking all the above considerations into account, a request for a combined Developmental Neurotoxicity study in rodents (OECD TG 426) and Neurotoxicity study in rodents (OECD TG 424), using rats with oral route of administration via feed, including the additional investigations in the OECD TG 424 part of the study (as specified in Appendix 3) is the most appropriate request to clarify the current concerns.

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Consideration of your comments on the draft decision:
1. You have emphasized that Ziram does not produce Parkinson´s disease (PD)-like symptoms in any of the available studies like bradykinesia (reduction of motor activity), and that neither the 2-generation study ([redacted]) nor the DNT study ([redacted]) have shown a reduction of locomotor activity, but showed an increase of motor activity in the DNT study in high dose offspring. You have added that the increase in locomotor activity is the opposite of what could be expected from Parkinson's Disease and is not a neurotoxic effect but likely reflects the growth retardation of the pups (general toxicity) and concluded that Ziram does not induce bradykinesia in pups upon perinatal exposure.

ECHA does not agree that the lack of bradykinesia (reduction of motor activity) in offspring in the DNT and the 2-generation study can be used to negate a concern for parkinsonian disorders.

In the DNT study, the offspring have only been exposed perinatally through maternal dosing. Parkinsonian and other neurodegenerative disorders are diseases in adults, and the relevant timeperiod to investigate the relevant endpoints would be in adult animals after prolonged dosing during the adult part of their life. Therefore, a lack of decreased motor activity in young offspring after perinatal exposure cannot be used to negate a concern for parkinsonian disorders in adults.

In the 2-generation study ([redacted]), the F2 offspring were tested for effects on motor activity at PND 13, 17, 21 and 60. Thus the offspring were relatively young adults at the time of the last motor activity test (sexual maturation in male rats usually occurs around day 43-45). Further, the actual exposure of the offspring during lactation is uncertain and to infer an exposure equal to the dams would have to be supported by toxicokinetic evidence, which is for the moment lacking. Accordingly, the maximum period with direct dosing before testing the motor activity, was from weaning at PND 22 to PND 60, i.e. 38 days. As highlighted in the scientific opinion from the EFSA PPPR Panel (EFSA 2017), even if relevant endpoints for investigation of parkinsonian disorders are included in a guideline study, severe adverse effects may still be missed since the effects will only be observed after exposure for a prolonged period of time.

When taking into account the short period of direct dosing and the relatively young age at the time of the last activity measurement, it cannot be assumed that effects related to parkinsonian disorders in the F2 animals could be detected.

In addition, it is highlighted in the scientific opinion from the EFSA PPPR Panel (EFSA 2017) that a change in motor activity would only be identifiable in standard OECD 90-day guideline studies if neuronal loss in the nigrostriatal pathway was above the threshold activating motor deficits (i.e. neuronal cell loss above 30-70% would be needed to detect changes in motor activity, depending on the number of animals included in the investigations).

Accordingly, a study with a prolonged dosing period in adult animals (the dosing period of the F0 animals in the OECD TG 426 part of the study and 90 days thereafter in the OECD TG 424 part of the study) with the relevant histopathological and behavioural investigations, as detailed in the scientific opinion from the EFSA PPPR Panel (EFSA 2017), has been requested.

Further, in addition to the concern for parkinsonian disorders, there is a concern for

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developmental neurotoxicity and it is agreed that there is an increase in motor activity after exposure in the DNT study ([redacted]).

In addition, the concern for DNT does not exclude the concern for parkinsonian disorders. Ziram may act through more than one mode of action leading to several effects during the development and lifetime of an organism. In this case, there is a concern for developmental neurotoxicity due to the increased motor activity observed in the offspring after exposure in utero (in [redacted] and non-significantly in [redacted]), and there is a concern for parkinsonian disorders, based on the epidemiological and mechanistic studies, as laid out in this decision.

ECHA does not agree with you that the increase in locomotor activity observed in the [redacted] study is not a neurotoxic effect but likely reflects the growth retardation of the pups (general toxicity).

The significantly increased motor activity levels were observed at all timepoints in the highest dose group (PND 13, 17, 21 and 61) and at the two latest time points (PND 21 and 61) in the middle dose group. ECHA acknowledges that some maternal toxicity and lower pup weight was observed in the highest dose group, and that the observations in this dose group may be affected by delayed development of the pups. However, in the middle dose group no maternal toxicity was observed and a lower pup body weight was only observed in the males at one timepoint (PND 21). Therefore, the findings of increased motor activity at the two last time points in the middle dose group cannot be dismissed due to maternal toxicity or delayed development in the pups. The concern for DNT effects is further supported by non-significant increases in motor-activity in the F2 offspring in the 2-generation toxicity study ([redacted]). This study applied only 10 animals/sex/group (compared to 20 animals/sex/group in the [redacted] DNT study), and although the observed tendency was not statistically significant, this finding corroborates the statistical significant results by [redacted].

2. Furthermore you stressed that the 90-day neurotoxicity study with Ziram ([redacted]) failed to show effects on locomotor activity, and that there was no effects on rotarod performance, which is an important readout for PD-studies in the rat.

There seems to be some confusion regarding study references. The 90-day neurotoxicity study referred to in your comments to the Draft decision as '[redacted]', is in your reference list referred to as '[redacted]', but it is believed that it is indeed the same study as the one referred to in this decision and the Ziram pesticide DAR (DAR 2004) as '[redacted], as the two reference seem to cover the same study:

- In your comments to the revised draft decision (letter from [redacted] to ECHA): "[redacted]".
- In this decision (and the Ziram pesticide report (DAR 2004)): "[redacted]".

In the Registration dossier, there is no information about rotarod testing in this study ([redacted]). It is therefore not clear to ECHA at which timepoint the rotarod testing was conducted, how the testing was conducted or how many animals

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were tested per dose group (the power of the test to detect changes is unknown).

During the process of substance evaluation, the evaluating MSCA had in the initial draft decision in 2013 proposed to request a DNT study (OECD TG 426). You provided comments to this request and submitted a DNT study which had already been performed but not included in the Registration dossier. The study was then subsequently included in the Registration dossier. At that point in time, the evaluating MSCA urged the Registrants to include all relevant information for the ongoing substance evaluation in the Registration dossier, as they are obliged to under REACH. This was apparently not followed by the Registrants, as new information from the testing ([redacted]) was brought forward, which is not included in the Registration dossier in sufficient detail to assess the data. In order to properly interpret the results from this previously performed rotarod study, a full study report showing detailed description of methods, individual animal data and group mean results, would be needed.

In any case, even if no effect on performance in a rotarod assessment after 90 days exposure is observed in the [redacted] study, it would in not dismiss the concern for parkinsonian disorders. The investigation of the rotarod measurements is only one parameter out of numerous needed/possible to investigate effects linked to parkinsonian disorders, with the specific sectioning of the brain and staining of special areas with relevant staining methods being the most sensitive and important. As pointed out in the scientific opinion from the EFSA PPPR Panel (EFSA 2017), degeneration of DA neuronal cells of the nigrostriatal pathway should on its own be considered as an adverse outcome, and is more sensitive to exposure to substances inducing parkinsonian disorders than motor activity effects. It is highlighted that a change in motor activity would only be identifiable in standard OECD 90-day guideline studies if neuronal loss in the nigrostriatal pathway was above the threshold activating motor deficits (i.e. neuronal cell loss above 30-70% would be needed to detect changes in motor activity, depending on the number of animals included in the investigations).

ECHA therefore does not agree that the lack of effects in one rotarod test (even if conducted properly and with an adequate number of animals per dose group), would dismiss the concern for parkinsonian disorders.

3. Concerning the epidemiological studies cited in the draft decision they seem to describe an enhanced risk for Parkinson's Disease. The risks were described for Ziram with co-exposure to paraquat. You pointed out that the OR for Parkinson's Disease in the absence of paraquat exposure was not statistically significant, and that the association between Ziram and Parkinson's Disease was statistically significant only if Ziram was reported both from residential and workplace sources. You stressed that the case numbers were very small, making the association non-robust.

There are two epidemiological studies showing associations between Ziram exposure and development of Parkinson's Disease. In Fitzmaurice et al., 2014, the association between Ziram and Parkinson's Disease was statistically significant when both residential and workplace exposure to Ziram was reported. The OR was also above 1 when only workplace exposure was reported, but this was not statistically significant. In Wang et al., 2011, the OR was above 1 for both occupational exposure alone and residential exposure alone. In this publication, statistics seem not to be performed.

It is acknowledged that the number of cases being exposed to only Ziram were small in the two publications and this is a significant limitation of the studies. It is also

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acknowledged that this may lead to possible false positive associations – in reality risk inflation - but also it can be argued that the finding of associations is more significant when the number of included cases is small, since the power of the study decreases. For the time being this is an uncertainty of the particular studies. However, it is noteworthy that ORs above 1 are found for associations between Ziram exposure and development of Parkinson's Disease in two different investigations, which strengthens the robustness of the association, although based on the same dataset.

In addition, it is acknowledged that co-exposure to other substances may occur, as also other confounders like recall bias may play a role. This is a draw-back of epidemiological studies in general, and therefore the epidemiological studies are not used alone as ground for concern. Together with the mechanistic studies reviewed in the decision, ECHA finds that these epidemiological studies raise a concern for parkinsonian disorders, which is important to investigate further.

4. It is hypothesised that Ziram inhibits aldehyde dehydrogenase (ALDH) which interferes with detoxification of the toxic dopamine metabolite DOPAL, and that impaired detoxification leads to the death of dopaminergic neurons. You draw the attention to the fact that ALDH inhibition by Ziram is only 20% shown *ex vivo* in neuronal suspensions, and that an *in vivo* study on metabolism of Ziram in rats has shown that Ziram or its metabolites do not distribute to the brain as less than 0,01 % of the administered dose has been found in the brain (Cheng, 1989). You state that it therefore is unlikely that appreciable inhibition of brain neuronal ALDH can be achieved with Ziram–containing pesticides under realistic condition and is not a plausible key event or MIE (Molecular Initiating Event) for Ziram in the AOP towards parkinsonian motor deficits.

The evaluating MSCA and ECHA cannot find specific information on distribution of Ziram to the brain in the registration dossier.

In the Ziram pesticide report (DAR 2004), the following is stated: "Distribution has not been studied in details: quite low levels of radioactivity (1.1-0.01%) were retained 7 days after oral administration and localised in the excretion organs (liver, kidneys, lungs), the carcass and blood. Recovery of radioactivity was < 0.01% in bone, brain, fat, muscle, gonads, spleen, pituitary gland and thyroid gland. After repeated exposure, recovery of radioactivity in tissues and carcass was low 7 days after exposure, suggesting that the compound does not accumulate...(Cheng, 1989, task force study)."

However, even if Ziram does not seem to accumulate in the brain, sufficient amounts of Ziram could still pass through the brain and induce adverse effects. For instance evidence of thyroid effects of Ziram exist, even though less than 0,01 % of the administered dose has also been found in the thyroid in the [redacted] study. ECHA therefore does not agree that it is unlikely that appreciable inhibition of brain neuronal ALDH can be achieved under realistic conditions after exposure to Ziram.

5. You point out that all known chemical inducers of Parkinson's Disease cause Parkinson's Disease symptoms in adult rats, and that therefore the 90-day neurotoxicity study with Ziram in adult rats is an adequate test system for demonstrating the absence of Parkinson's Disease induction by Ziram due to its inclusion of rotarod measurements. You also point out that the DNT and 2-generation studies fail to show bradykinetic behaviour.

ECHA agrees that adult rats is the adequate test system to investigate effects linked to

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parkinsonian disorders. The evaluating MSCA and ECHA cannot find any information about the rotarod measurements in the registration dossier. As indicated in the response to comment no.2 above, there is no information about how the test was conducted or how many animals were tested per group (the power of the test to detect changes is unknown) and neither the results were reported.

In any case, even if no effect on performance in a rotarod assessment after 90 days exposure is observed in the [redacted] study, it would not dismiss the concern for parkinsonian disorders. The investigation of the rotarod measurements is only one parameter out of numerous needed/possible to investigate effects linked to parkinsonian disorders, with the specific sectioning of the brain and staining of special areas with relevant staining methods being the most sensitive and important. As pointed out in the scientific opinion from the EFSA PPPR Panel (EFSA 2017), degeneration of DA neuronal cells of the nigrostriatal pathway should on its own be considered as an adverse outcome, and is more sensitive to exposure to substances inducing parkinsonian disorders than motor activity effects. It is highlighted that a change in motor activity would only be identifiable in standard OECD 90-day guideline studies if neuronal loss in the nigrostriatal pathway was above the threshold activating motor deficits (i.e. neuronal cell loss above 30-70% would be needed to detect changes in motor activity, depending on the number of animals included in the investigations).

ECHA therefore does not agree that the lack of effects in one rotarod test (even if conducted properly and with an adequate number of animals per dose group), would dismiss the concern for parkinsonian disorders.

The results from the DNT study and the 2-generation study do not provide any useful information about the concern for parkinsonian disorders. The offspring in the DNT study have only been exposed to Ziram perinatally, through maternal dosing, i.e. they have had a short period of exposure during early development. Parkinsonian disorders are diseases in adults, and according to the scientific opinion from the EFSA PPPR Panel (EFSA 2017), the relevant timeperiod to investigate effects linked to parkinsonian disorders is in adult animals after prolonged dosing during the adult part of their life.

Based on the recommendations from the scientific opinion, it is requested that adult parental animals (F0) from the OECD TG 426 study are not terminated by the time of weaning but are rather used in the OECD TG 424 part of the study, which shall last for 90 days. This way, a prolonged dosing period of adult animals is obtained, which include the dosing period of the F0 animals in the OECD TG 426 part of the study followed by 90 days in the OECD TG 424 part of the study.

6. You have offered, without prejudice to the technical feasibility, to re-investigate brain sections from the existing Ziram DNT study ([redacted]) for potential effects on dopaminergic neurons in brains of Ziram-exposed pups.

Investigating offspring from the DNT study is not found to be an adequate model for assessing effects linked to parkinsonian disorders. The offspring in the DNT study have only been exposed to Ziram perinatally, through maternal dosing, i.e. they have had a short period of exposure during early development. Parkinson's Disease is a disease in adults, and according to the scientific opinion from the EFSA PPPR Panel (EFSA 2017), the relevant timeperiod to investigate effects linked to parkinsonian disorders is in adult animals after prolonged dosing during the adult part of their life. Based on the recommendations from the scientific opinion, ECHA has requested that adult parental

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animals (F0) from the OECD TG 426 study are not terminated by the time of weaning but are rather used in the OECD TG 424 part of the study, which shall last for 90 days. This way, a prolonged dosing period of adult animals is obtained, which include the dosing period of the F0 animals in the OECD TG 426 part of the study followed by 90 days in the OECD TG 424 part of the study.

7. You do not agree to conduct any new vertebrate study into a potential association between Ziram and Parkinson's Disease since you are of the opinion that no relevant effects can be discerned in the existing body of relevant and reliable studies that would necessitate further research.

ECHA does not agree that there are no relevant effects in the existing body of literature that necessitate further investigations. To the contrary, the available literature raise two concerns: A concern for developmental toxicity and a concern for parkinsonian disorders, as laid out in this decision.

Consideration of the PfAs and your comments on the PfAs

A proposal for amendment (PfA) disagreed with new animal testing being necessary at this stage: Regarding the concern for developmental neurotoxicity, the PfA stated that there is sufficient data already available to make a robust assessment of this endpoint. It is recognized by the PfA submitter that in the [redacted] study, certain endpoints were not studied (startle response; cognitive function), but it is argued that when considered alongside the [redacted] study, these studies do not provide any convincing evidence that Ziram is a developmental neurotoxicant, and that this is in agreement with the EFSA conclusion (EFSA 2017).

Regarding the concern for parkinsonian disorders, the PfA submitter pointed out that the epidemiological studies described in the draft decision have a number of limitations and do not demonstrate a causal relationship between Ziram exposure and Parkinson's Disease. Further, the PfA pointed out that the reported clinical signs in the animal studies cited in the SEv report are inconsistent with clinical signs reported in animal models of Parkinson's disease.

In reply to the PfA regarding the concern for developmental toxicity, it is considered that the studies by [redacted] indicate that Ziram may cause adverse effects on the developing nervous system manifested as increased activity before weaning and in adulthood, and this raises a concern for developmental neurotoxicity. Therefore, further investigation is warranted in order to address this concern. The scientific opinion from the EFSA PPPR Panel (EFSA 2017) did not discuss developmental neurotoxicity effects of Ziram.

In reply to the concern for parkinsonian disorders, ECHA agrees that there are a number of limitations in the epidemiological studies, which shows an association between pesticide exposure and the development of Parkinson's Disease in humans. This is also supported by the scientific opinion from the EFSA PPPR Panel (EFSA 2017). It is, however, the view of ECHA that the epidemiological studies indicate an association between exposure to Ziram and development of Parkinson's Disease, and that the epidemiological studies together with the mechanistic studies raise a concern that Ziram exposure may induce parkinsonian disorders. Since no final conclusions can be drawn based on the available epidemiological and mechanistic studies, further testing is requested to clarify the concern.

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Further, the available chronic/repeated dose rodent studies available have been thoroughly evaluated for clinical signs of parkinsonian motor deficits. No signs of parkinsonian disorders were observed in these studies. However, none of these studies were designed to investigate neurodegenerative effects, and they did not include the most sensitive and important endpoints for investigation of development of parkinsonian disorders (i.e. specific sectioning of the brain and staining of special areas with relevant staining methods).

In the DNT study [redacted], the offspring have only been exposed perinatally through maternal dosing. Parkinsonian and other neurodegenerative disorders are diseases in adults, and the relevant timeperiod to investigate the relevant endpoints would be in adult animals after prolonged dosing during the adult part of their life. Therefore, a lack of decreased motor activity in young offspring after perinatal exposure cannot be used to negate a concern for parkinsonian disorders.

The limitations of the 2-generation study [redacted] are discussed above under *"Consideration of your comments on the draft decision"*, point 1.

Another PfA questioned whether the requested investigations in rat will provide reliable and relevant information on the potential of Ziram to cause Parkinson's disease.

It is the view of ECHA that the requested combined study may inform about developmental neurotoxicity in the TG426 part of the study. Further, the TG424 part of the study aims to inform about neurotoxic effects including effects linked to parkinsonian disorders since it includes a range of endpoints relevant to investigate parkinsonian disorders (i.e. specific investigations of relevant brain areas using special immunohistochemical approaches instead of standard haematoxylin and eosin (H&E) staining as well as special behavioural tests). The endpoints included were chosen based on recommendations from the scientific opinion from the EFSA PPPR Panel (EFSA 2017).

Accordingly, a study with a prolonged dosing period in adult animals (the dosing period of the F0 animals in the OECD TG 426 part of the study and 90 days thereafter in the OECD TG 424 part of the study) with the relevant histopathological and behavioural investigations, as detailed in the scientific opinion from the EFSA PPPR Panel (EFSA 2017), is requested.

In your comments on the PfAs, you provide a position paper summarising the available data relevant to neurotoxicity. This data has been considered, as explained above. You repeat your view given in your comments on the draft decision, that Ziram does not cause relevant neurodegenerative effects and the requested combined OECD 426/OECD 424 study is not justified. Full reasoning for the requested study is provided above.

Conclusion

Therefore, based on the substance evaluation and pursuant to Article 46(1) of the REACH Regulation, ECHA concludes that you are required to carry out the following study using the registered substance, Ziram, subject to this decision: Combined Developmental Neurotoxicity study (OECD TG 426) and Neurotoxicity study in rats (OECD TG 424), oral route of administration via feed, including additional investigations in the OECD TG 424 part of the study, as specified in Appendix 3.

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Appendix 2: Procedural history

On the basis of an opinion of the ECHA Member State Committee and due to initial grounds for concern relating to possible endocrine disrupting properties, and risk characterisation ratios (RCRs) in the close vicinity of 1 (workers), which indicates a need of clarification and possible reduction of exposure, Ziram CAS No 137-30-4 (EC No 205-288-3) was included in the Community rolling action plan (CoRAP) for substance evaluation to be evaluated in 2012. The updated CoRAP was published on the ECHA website on 29 February 2012. The Competent Authority of Denmark (hereafter called the evaluating MSCA) was appointed to carry out the evaluation.

Pursuant to Article 45(4) of the REACH Regulation the evaluating MSCA carried out the evaluation of the above substance based on the information in your registration(s) and other relevant and available information. A draft decision was then sent to you on 4 April 2013 proposing the following information requests: 1. Developmental Neurotoxicity Study, in rats using oral exposure (Developmental Neurotoxicity Study, OECD 426) and 2. Information in the Chemical Safety Reports (CSRs) including: a. Justification that risks to workers are adequately controlled (Annex I, 6.4 of the REACH Regulation; section 10 of the CSR); b. Revision of the DNEL derivation, based on dermal absorption and of the exposure estimations accordingly; c. Full documentation that all Ziram is consumed in the described production processes to ensure that consumers and downstream users are not exposed to residual free Ziram.

You provided comments on this draft decision on 5 May 2013. The evaluating MSCA considered these comments and the updated registration dossiers you submitted on 10 July 2013 and 12 March 2015. In the updated registration dossier you provided supplementary data on exposure. The provided data was sufficient enough for the evaluating MSCA to withdraw the initial requests based on the concerns related to human exposure. Thus the concern on the exposure was clarified and the RCR consequently reduced. Furthermore, a study corresponding to the requested study (Developmental Neurotoxicity Study, OECD 426) had been performed, and you have now included it in the updated registration dossier.

Based on the information in the updated dossier the evaluating MSCA concluded, that Ziram may cause adverse effects on the developing nervous system manifested as increased activity before weaning and in adulthood, but no final conclusions could be drawn based on the available studies. The evaluating MSCA had a residual concern that these adverse effects on the developing nervous system could be induced by an endocrine disrupting mode of action (MoA) (thyroid disruption). However, as Ziram may react via more than one MoAs), the evaluating MSCA considered it to be very uncertain if further testing in order to clarify a potential endocrine MoA would lead to improved risk management.

In addition, in the course of the evaluation the evaluating MSCA identified specific concerns regarding involvement of Ziram in the development of parkinsonian disorders in papers from the public literature. Consequently the evaluating MSCA considered that a revised testing strategy was required to clarify the concerns for developmental neurotoxicity and parkinsonian disorders.

Therefore, it prepared a revised draft decision pursuant to Article 46(1) of the REACH Regulation with further information requests, which replaced the previous draft decision

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dated 4 April 2013. It submitted the draft decision to ECHA on 21 June 2017, which was sent to you for your comments on 17 July 2017.

By 23 August 2017 ECHA received comments from you and forwarded them to the evaluating MSCA without delay.

The evaluating MSCA took into account your comments, which were sent within the commenting period, and they are reflected in the reasons (Appendix 1). The request and the deadline were not amended.

Proposals for amendment by other MSCAs and ECHA and referral to the Member State Committee

The evaluating MSCA notified the draft decision to the competent authorities of the other Member States and ECHA for proposal(s) for amendment.

Subsequently, the evaluating MSCA received proposals for amendment to the draft decision and modified the draft decision. They are reflected in the reasons (Appendix 1).

ECHA referred the draft decision, together with your comments, to the Member State Committee.

ECHA invited you to comment on the proposed amendments.

Your comments on the proposed amendments were taken into account by the Member State Committee.

MSC agreement seeking stage

The Member State Committee reached a unanimous agreement on the draft decision during its MSC-57 meeting and ECHA took the decision according to Article 52(2) and 51(6) of the REACH Regulation.

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Appendix 3: Further information, observations and technical guidance

1. This decision does not imply that the information provided by you in the registration(s) is in compliance with the REACH requirements. The decision neither prevents ECHA from initiating compliance checks on your dossier(s) at a later stage, nor does it prevent a subsequent decision under the current substance evaluation or a new substance evaluation process once the present substance evaluation has been completed.

2. Failure to comply with the request(s) in this decision, or to fulfil otherwise the information requirement(s) with a valid and documented adaptation, will result in a notification to the enforcement authorities of your Member State.

3. In relation to the required experimental study/ies, the sample of the substance to be used shall have a composition that is within the specifications of the substance composition that are given by all Registrant(s). It is the responsibility of all the Registrant(s) to agree on the tested material to be subjected to the test(s) subject to this decision and to document the necessary information on composition of the test material. The substance identity information of the registered substance and of the sample tested must enable the evaluating MSCA and ECHA to confirm the relevance of the testing for the substance subject to substance evaluation.

   In relation to the experimental stud(y/ies) the legal text foresees the sharing of information and costs between Registrant(s) (Article 53 of the REACH Regulation). You are therefore required to make every effort to reach an agreement regarding each experimental study for every endpoint as to who is to carry out the study on behalf of the other Registrant(s) and to inform ECHA accordingly within 90 days from the date of this decision under Article 53(1) of the REACH Regulation. This information should be submitted to ECHA using the following form stating the decision number above at:
   https://comments.echa.europa.eu/comments_cms/SEDraftDecisionComments.aspx

   Further advice can be found at
   http://echa.europa.eu/regulations/reach/registration/data-sharing.
   If ECHA is not informed of such agreement within 90 days, it will designate one of the Registrants to perform the stud(y/ies) on behalf of all of them.

4. In relation to the requested study, the following technical guidance is provided:

   The request is for a combined oral Developmental Neurotoxicity study (OECD TG 426) and Neurotoxicity study in rodents (OECD TG 424), using rats with oral route of administration via feed, including additional investigations in the OECD TG424 part of the study, using the registered substance.

   The Developmental Neurotoxicity Study in rodents: OECD TG 426, using rats with oral route of administration via feed, shall be conducted according to the guideline. The adult parental animals from this study (F0) shall be kept and tested according to a Neurotoxicity study: OECD TG 424 with additional investigations, as specified below.

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(A) Number and sex of animals:

At least 20 F0 dams and 20 F0 males shall be included in the OECD TG 426 part of the study in order to produce 20 litters per dose group.

After mating (for the males) and after weaning (for the females), 10 F0 animals/sex per group shall be randomly chosen to continue in the part of the study to be conducted according to the OECD TG 424. At the end of the study, all 10 animals/sex per group shall be perfused in situ and used for detailed neurohistopathology.

Reasoning:
In the OECD TG 426, a total of 20 litters is recommended in each dose group. Thus, at least 20 F0 dams and 20 F0 males shall be included in the study.

After mating (for the males) and after weaning (for the females), 10 F0 animals/sex per group shall be randomly chosen to continue in the part of the study to be conducted according to the OECD TG 424.

According to TG 424, at least 10 males and 10 females should be included in each group, and at least 5 males and 5 females should be perfused in situ and used for detailed neurohistopathology at the end of the study.

In order to increase the sensitivity of the specific investigations for endpoints related to parkinsonian disorders, all 10 animals/sex per group shall be perfused in situ and used for detailed neurohistopathology at the end of the study. This increases the power in the neurohistopathological investigations, which are particularly relevant for detection of effects linked to parkinsonian disorders.

(B) Dose level setting:

In both the TG 424 and TG 426 study, dose levels of (or similar to): 0, 100, 200 and 400 ppm Ziram in the feed shall be investigated. Importantly, the feed concentrations must correspond to doses of 0, 6-9, 15-20 and 30-40 mg/kg bw/day in adult (non-lactating) animals. Doses in mg/kg bw/day during lactation will inevitably be higher than this, which is not a problem. It is however imperative that the lowest dose is kept below 10 mg/kg bw/day in the adult parental males and females tested in the OECD TG 424 part of the study. In order to respect this request, it may be necessary to perform a range-finding study, for calculating the exact concentrations of Ziram which shall be included in the feed, in the combined TG 424/426 study.

Reasoning:
Previous repeated dose studies investigating neurotoxicity, developmental- and reproductive toxicity have investigated dose levels of 0, 72, 207 and 540 pm. In the performed 2-generation study the highest dose of 540 ppm was too high, and had to be reduced to 340 ppm during lactation. In the developmental neurotoxicity study by [redacted]) the highest dose caused some maternal toxicity (15 % decrease in weight during the last part of gestation). The dose of approximately 400 ppm for the requested study might still exert some degree of maternal toxicity, but it is predicted not to cause toxic effects in a degree that it will be necessary to be decreased during the course of the study. In the previously performed 90-day study ([redacted]), the

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dose of 540 ppm (34 mg/kg bw/day) also caused a significant reduction in neuropathy target esterase (NTE), a protein which plays an important role in neural development. It will therefore be relevant to investigate whether this effect will also be present at the 400 ppm dose.

The lower doses of 200 and 100 ppm were chosen by applying a reduction factor of 2. The chosen middle dose of 200 ppm is furthermore similar to the dose of 15 mg/kg bw/day, which showed indications of neurological effects after just a single dose in the 90-day acute neurotoxicity study by [redacted]. In addition, the lowest dose of 100 ppm was chosen to represent a dose that is below 10 mg/kg bww/day, in order to assess whether the registered substance can be classified STOT RE 1, depending on the results of the requested study.

The dosing of the animals with Ziram, is to be via the feed (and not via gavage) in order to minimize handling of the animals. This is especially important during the behavioural studies which are to be performed in both the TG 426 and TG 424 study. Analysis of the feed, in order to validate the concentration and the stability of Ziram, is necessary, and should be performed according to relevant procedures /guidelines.

(C) Dosing period:

In the OECD TG 426 part of the study, the dosing period shall follow the guideline. The TG 424 part of the study shall be initiated (using the F0 animals from the OECD TG 426 part of the study) after weaning of the offspring in the OECD TG 426 part of the study and shall last 90 days. The OECD TG 424 part of the study shall be initiated at the same point of time for both males and females.

Dosing of the F0 animals shall be continued throughout the period, also if the F0 animals are kept for a period of time after end of the OECD TG 426 part of the study and before initiation of the OECD TG 424 part of the study.

Reasoning:
It is highlighted in the scientific opinion from the EFSA PPR Panel that even if relevant endpoints for investigation of effects linked to parkinsonian disorders are included in a guideline study, severe adverse effects may still be missed since the effects will only be observed after exposure for a prolonged period of time (EFSA 2017). In the TG 424, a dosing regimen of 28 days, 90 days or 1 year or longer is suggested. No exact advice on adequate exposure period is available, but weighing the outcome of the scientific opinion from the EFSA PPR Panel against animal welfare and resource considerations, a dosing period of 90 days shall be used in the OECD TG 424 part of the study (in addition to the dosing period of the F0 animals during the OECD TG 426 part of the study).

(D) Functional tests

D1) Timing of functional tests
The first assessments shall be performed in the F0 animals from the OECD TG 426 study, prior to initiation of Ziram exposure. The following assessments shall be performed during the 90 days of the OECG TG 424 study (i.e. after weaning of the offspring in the OECD TG 426 part of the study and initiation of the OECD TG 424 part of the study).

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Reasoning:
According to OECD TG 424, assessment of functional tests in a 90-day study shall be performed prior to first exposure, and then again once during the 1st or 2nd week of exposure and monthly hereafter.

D2)Functional tests to be included in the OECD TG 424 part of the study.

The following functional tests shall be conducted in order to investigate endpoints linked to parkinsonian disorders:

D2A) Examination of co-ordination and balance by rotarod test and pole test.
Rotarod: assessment of motor coordination. The animals are placed on a rotating rod that is subjected to linear acceleration. The latency to fall from the rod is detected (Kallai et al., 2017, Rozas et al., 1997, EFSA 2017).

Pole test: the animal is placed on a gauze-taped pole with the head upwards below the top of the pole. Two parameters are detected: 1) time until animals turn by 180°; 2) time until the animals reach the floor (Rauch et al., 2010, EFSA 2017).

D2B) Examination of motor coordination and synchrony during normal walking by stride length tests. Ink is applied to the paws (one color for front paws and one color for hind paws) and the rats walk across a blank sheet of ink-absorbent paper in a narrow walkway. The distance between single steps on each side are measured (Mullenix et al., 1975, Klapdor et al., 1997, Sedy et al., 2008, EFSA 2017).

Reasoning (D2A-D2B):
The OECD TG 424 states that the functional tests shall include assessment of sensory reactivity to stimuli of different modalities [e.g., auditory, visual and proprioceptive stimuli], assessment of limb grip strength and assessment of motor activity, measured with an automated device]. It is also stated that if there are other data to indicate potential neurotoxic effects, the inclusion of more specialized tests should be considered.

In the present case there is a concern regarding parkinsonian disorders. As symptoms of parkinsonian disorders in rodent models are best assessed by investigating motor deficits such as coordination, balance and gait abnormalities, which are affected by degeneration of dopaminergic neurons in substantia nigra pars compacta and striatum (EFSA 2017), the functional tests mentioned in D2A-B shall be conducted since they are specifically directed towards investigating these endpoints.

(E) Histopathology

In addition to the histopathological investigations included as mandatory in the OECD TG 424, the following histopathological methods and investigations shall be included in order to investigate endpoints linked to parkinsonian disorders. In respect to the mandatory histopathological investigation presented in the test guideline, and the investigations presented below, highest priority should be given to the investigations specified below

E1)
Appropriate samples of the brain shall be obtained (i.e. rostral midbrain section

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through the anterior colliculus) (EFSA 2017).

Reasoning:
Pathologically, parkinsonian disorders are characterised by the loss of dopaminergic neurons in the substantia nigra pars compacta and the loss of dopamine in the striatum (EFSA 2017). Morphological assessment of brain structures is a standard requirement in the regulatory toxicological studies supporting the risk assessment of chemical substances and it is a regulatory expectation that the anatomical structures belonging to the nigrostriatal pathway would be included and evaluated as part of the standard evaluation of the brain. According to OECD TG 424 and TG 426, brains shall be weighed and histopathological examination performed in the brain, spinal cord and peripheral nerves. In both these studies, adequate samples from all major brain regions shall be taken to ensure a thorough examination. In order to investigate effects of Ziram exposure linked to parkinsonian disorders, extra care shall be taken to assure that appropriate samples of the brain are obtained (i.e. rostral midbrain section through the anterior colliculus).

E2) Degeneration of dopaminergic neurons of the nigrostriatal pathway shall be investigated.

Detailed neuropathology assessment of the substantia nigra pars compacta (SNpc) and striatum shall be conducted with inclusion of:

E2A) Neuronal cell loss and neuronal cell damage.

Investigation of neuronal cell loss and neuronal cell damage in the substantia nigra pars compacta and the striatum by use of a special stain procedure (the silver degeneration stain (Switzer, 2000, Betarbet et al., 2000) and/or the Fluoro Jade stain (Fornai et al., 2003, Betarbet et al., 2000, Schmued et al., 1997, Schmued and Hopkins, 2000)) in addition to Hematoxylin and Eosin stain. The assessment of neuronal cell loss must include carefully conducted stereology (not simply morphometry) and pathology studies (Smeyne et al., 2016, Breckenridge et al., 2013, EFSA 2017). If needed, methods shall be adapted to conductance in rats.

E2B) Total number of dopaminergic neurons.

Investigation of the total number of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and in the striatum by detection of the total number of Tyrosine hydroxylase- (the enzyme responsible for catalysing the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine) positive neurons in SNpc and in the striatum by immunocytochemistry followed by cell counting. Stereological protocols for cell counting shall be applied (Fettissov et al., 1999, Breckenridge et al., 2013, Smeyne et al., 2016, EFSA 2017). If needed, methods shall be adapted to conductance in rats.

E2C) Detection of dopamine neuron terminals.

Antibody mediated staining of the dopamine transporter (DAT) in DA neuronal terminals in the substantia nigra pars compacta (SNpc) and in the striatum shall be performed. The dopamine transporter shall be visualized and quantified using antibody mediated staining in tissue slices (Fornai et al., 2003, EFSA 2017).
Reasoning (E2A+B+C):

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Death of dopaminergic neurons of the nigrostriatal pathway with decreased dopamine is an essential step in the pathway lading to parkinsonian disorders, since this event is essential for motor control. Also in humans, degeneration of dopaminergic neurons within the substantia nigra pars compacta is regarded as a key event in parkinsonian disorders and in in a quantitative manner directly linked to the occurrence of clinical signs indicative of parkinsonian disorders. The severity of the clinical signs in humans correlates with the degree of nigral cell loss, and the reduced level of dopamine in the striatum. Due to the limitations in the assessment of moderate motor impairment of rodents and the well-established correlation between striatal dopamine content and impaired motor output, analysis of striatal dopamine can be used as a surrogate readout for the assessment of motor deficits. As alternative to the detection of striatal dopamine that is to a large extent limited to live detection setups due to its instability in tissues, the number of remaining dopamine neurons in the substantia nigra pars compacta has been used as an alternative endpoint. Thus, degeneration of dopaminergic neurons of the nigrostriatal pathway in rodents is considered an adverse effect in the regulatory framework, even in the absence of clear clinical symptoms or motor deficits (EFSA 2017).

Reasoning (E2A):
The special stain procedure shall be used since degenerating and/or degenerated neurons can be detected by the silver stains and the Fluoro Jade stains, but will be missed by the use of a normal H/E stain (EFSA 2017). The silver degeneration stain is considered the best method to trace degeneration of axons. By this matter, products from disintegrated cells are visualized (Switzer, 2000; Betarbet et al., 2000). Fluoro Jade stain is a fluorochrome derived from fluorescein used in neuroscience disciplines to label degenerating neurons. It is an alternative technique to the traditional methods and may be preferred due to its simplicity of staining procedures. However, the mechanism by which it labels degenerating neurons is unknown (Betarbet et al., 2000, Schmued et al., 1997).

Reasoning (E2B):
Thyrosin hydroxylase (TH) is a marker specific for dopaminergic neurons and the quantification of TH-positive cells can be used to investigate effects on the number of dopaminergic neurons (EFSA 2017). Ziram has been shown to decrease TH-positive cells in rat primary mesencephalic cell cultures in vitro (Chou et al., 2008), and this endpoint *in vivo* is therefore of high relevance when investigating effects of Ziram exposure linked to parkinsonian disorders.

Reasoning (E2C):
In some specific cases thyrosin hydroxylase (TH) expression has been observed to be of limited suitability for assessment of dopaminergic neuronal numbers. Staining of dopaminergic neuronal terminals in the striatum is therefore by some suggested to be a more reliable indirect marker for striatal dopamine content. For the analysis of nigrostriatal terminals, the dopamine transporter (DAT) can be visualised, for example by antibody mediated staining in tissue slices (Fornai et al., 2003, EFSA 2017). Even though Ziram has not previously been shown to affect this endpoint, it shall be assessed since it may be a more reliable marker for striatal dopamine content than TH.

Reasoning (E2A+B+C):
Stereological protocols for cell counting shall be applied since this is the most

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sensitive method to confirm neurodegeneration quantitatively (Breckenridge et al., 2013, Smeyne et al., 2016, EFSA 2017).

E3) Investigation of impaired proteostasis.

Signs of impaired proteostasis shall be investigated by detection of levels of α-synuclein and ubiquitin in the subtantia nigra pars compacta (SNpc) and in the striatum. This shall be evaluated in fixed tissue and quantified for fluorescence intensity (Betarbet et al., 2000 and 2006, Kuusisto et al. 2003, EFSA 2017).

Reasoning:
Impaired proteostasis is a key event leading to degeneration of dopaminergic neurons of the nigrostriatal pathway (EFSA 2017). Proteostasis regulation is the main defence mechanism against toxic proteins, whose accumulation could greatly compromise normal cellular function and viability. Therefore, the chaperone and degradation systems assuring the removal of misfolded and aggregated proteins, as well as damaged, dysfunctional cellular organelles, play a key role in cellular homeostasis. The two major degradation systems are the ubiquitin–proteasome system (UPS) and the autophagy–lysosome pathway (ALP). The UPS works through the attachment of multiple ubiquitin molecules to a protein substrate, followed by the subsequent degradation of the tagged polyubiquitinated protein by the proteasome. A compromised function of the UPS leads to the accumulation of ubiquitylated proteins, such as α-synuclein, and one of the main aggregated proteins found to accumulate in nigrostriatal cells during parkinsonian disorders is α-synuclein (EFSA 2017). Ziram has been shown to inhibit the UPS and increase α-synuclein levels in vitro (Chou et al., 2008, Lulla et al., 2016), and investigation of levels of α-synuclein and ubiquitin in the relevant brain areas in vivo is therefore of high relevance when investigating effects of Ziram exposure linked to parkinsonian disorders.

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