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Risk assessment of nitrate and nitrite in feed

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Abstract

The European Commission asked EFSA for a scientific opinion on the risks to animal health related to nitrite and nitrate in feed. For nitrate ion, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) identified a $BMDL_{10}$ of 64 mg nitrate/kg body weight (bw) per day for adult cattle, based on methaemoglobin (MetHb) levels in animal's blood that would not induce clinical signs of hypoxia. The $BMDL_{10}$ is applicable to all bovines, except for pregnant cows in which reproductive effects were not clearly associated with MetHb formation. Since the data available suggested that ovines and caprines are not more sensitive than bovines, the $BMDL_{10}$ could also be applied to these species. Highest mean exposure estimates of 53 and 60 mg nitrate/kg bw per day in grass silage-based diets for beef cattle and fattening goats, respectively, may raise a health concern for ruminants when compared with the $BMDL_{10}$ of 64 mg nitrate/kg bw per day. The concern may be higher because other forages might contain higher levels of nitrate. Highest mean exposure estimates of 2.0 mg nitrate/kg bw per day in pigs' feeds indicate a low risk for adverse health effects, when compared with an identified no observed adverse effect level (NOAEL) of 410 mg nitrate/kg bw per day, although the levels of exposure might be underestimated due to the absence of data on certain key ingredients in the diets of this species. Due to the limitations of the data available, the CONTAM Panel could not characterise the health risk in species other than ruminants and pigs from nitrate and in all livestock and companion animals from nitrite. Based on a limited data set, both the transfer of nitrate and nitrite from feed to food products of animal origin and the nitrate- and nitrite-mediated formation of N-nitrosamines and their transfer into these products are likely to be negligible.

Keywords: Nitrate, Nitrite, methaemoglobin, occurrence, exposure, feed, animals

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Amendment: The reference Lammarino has been corrected to Iammarino on page 12 and in the references list. To avoid confusion, the original version of the Scientific Opinion has been removed from the EFSA Journal, but is available on request, as is a version showing all the changes made.

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Summary

Following a request from the European Commission, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) assessed the risk to animal health related to the presence of nitrate and nitrite, in feed. The previous risk assessment from the CONTAM Panel on nitrite as an undesirable substance in feed (2009) has been re-evaluated in the light of the current requirements on data quality. The CONTAM Panel assessed nitrate and nitrite as ions.

Nitrates are generally highly soluble in water and play a substantial role as nutrients for plants used for feeds. In veterinary medicine, potassium nitrate is used as diuretic in pigs, cattle and horses. It is also used as a vasodilator, bronchodilator and as an antidote for cyanide poisoning. Sodium nitrite is an authorised feed additive (EU Register of Feed Additives pursuant to Regulation (EC) No 1831/2003). Under acidic conditions, nitrite could form N-Nitroso compounds (NOCs), including genotoxic and carcinogenic N-nitrosamines, when reacting with some secondary amines in the feed or endogenously in the stomachs of animals.

There is limited information on the absorption, distribution, metabolism and excretion (ADME) of nitrate and nitrite in farm and companion animal species. In ruminants, there is a rapid and dose-related absorption of nitrate and nitrite, with a complex interconversion between the two anions followed by a rapid excretion, mainly via urine. The main metabolic pathway in the rumen involves bacterial NADH- or FADH-nitroreductases mediating a two-step reduction of nitrate, first to nitrite and then to ammonia, which represents an important nitrogen source for bacterial protein synthesis. Nitrate reduction successfully competes with carbon dioxide reduction, limiting the biosynthesis of methane by the rumen bacteria, one of the most potent greenhouse gases.

In pigs, the extent of nitrate reduction to nitrite is much lower than in ruminants. The reduction occurs in the intestine, but also takes place in the oral cavity due to an extensive salivary recirculation.

Little is known about the kinetics of nitrate/nitrite in horses, in which nitrate reduction to nitrite is brought about by an active caecal and colonic microflora and is reportedly intermediate between ruminants and pigs. No relevant data on the kinetics of nitrate/nitrite in rabbits, poultry, dogs, cats, fur animals or fish have been identified in the literature.

The nitrate itself has a low order of toxicity compared to nitrite, the latter causing the formation of methaemoglobin (MetHb), a molecule with very limited oxygen carrying capacity. Methaemoglobinaemia is the major adverse effect resulting from MetHb formation. Interspecies differences in the rate of MetHb formation are mainly related to the extent and the rate of nitrate reduction to nitrite, which is highest in ruminants, lower in horses and lowest in the other monogastric species.

The mode of action (MoA) can be described for several effects of nitrate and nitrite in farmed and companion animals, such as increase in oxidative stress, the depression of thyroid function and the decrease in blood pressure. The MoA underlying other effects (vitamins A and E depletion, abortion and effects on fertility) are still to be unraveled.

The generation of MetHb, resulting from the reaction between nitrite and oxyhaemoglobin, is considered the mediator of most adverse effects following exposure to nitrate and nitrite in ruminants. However, studies to investigate the methane-reducing potential of nitrate in ruminant diets have demonstrated that feeding strategies (encapsulation, fractionation, even distribution and gradual exposure to nitrate in the diet) and ruminal adaptation to nitrate help to maintain asymptomatic MetHb levels.

In order to derive a reference point for nitrate in cattle that is protective for all feeding regimes, the CONTAM Panel considered oral dose-response studies involving direct feeding of nitrate, once a day, to non-adapted cattle, with post-prandial MetHb measurements.

New literature reviewed by the CONTAM Panel suggests that there is limited evidence for clinical signs occurring in most ruminant species and categories when MetHb levels remain below 10%. Therefore, this value was used to define the benchmark response for cattle. The CONTAM Panel calculated a $BMDL_{10}$ of 64 mg nitrate/kg body weight (bw) per day as the reference point for nitrate ion in adult cattle. Based on the literature reviewed, the $BMDL_{10}$ defined for adult cattle is also applicable for lactating cows and calves. However, the association of MetHb formation with reproductive effects in pregnant cows such as late abortions and still births has not been clearly demonstrated.

There was insufficient information to set a separate reference point for nitrate for ovines and caprines. They have not been demonstrated to be more sensitive to nitrate than bovines, and therefore, the $BMDL_{10}$ identified for adult cattle may also be applied for these animal species. The

CONTAM Panel could not identify any appropriate studies which could be used to determine reference points for nitrite in bovines, ovines or caprines.

In pigs, a dose of nitrate of 410 mg/kg bw per day and a dose of nitrite of 20 mg/kg bw per day do not induce clinical signs and can be considered as the reference points.

The CONTAM Panel could not identify any appropriate studies to establish a reference point for nitrate and nitrite in species other than ruminants and pigs.

The dietary exposure was estimated considering a final data set which contained 1,542 nitrate analytical data points for nitrate and 1,561 for nitrite. The data were sampled in 15 different European countries between 2010 and 2019 and were mainly reported by only three countries, while other countries submitted only a limited number of data sets. The highest mean nitrate concentrations were observed for the feed category 'forages and roughage, and products derived thereof', in particular for clover meal and lucerne, and for the feed 'tubers, roots, and products derived thereof', and in particular for potatoes. For categories with $\geq$ 5 analytical results, the highest nitrite mean concentrations were observed for the feed category 'tubers, roots, and products derived thereof', in particular for sugar beet molasses. Even higher nitrite mean concentrations were measured for 'other plants, algae and products derived thereof', in particular for sugar cane molasses, but this is based only on four analytical results available (of which two were left censored), and therefore should be considered only indicative. Estimates of exposure were hampered by the lack of data for many of the feeds commonly used in the diets of farmed and companion animals. Therefore, all exposure estimates are likely to be underestimated.

In ruminants, nitrate toxicity is most commonly reported in ruminants fed fresh herbage; however, due to the absence of any data on nitrate levels in fresh grass, it has not been possible to estimate exposure for those livestock most susceptible to nitrate toxicity. Due to insufficient data on levels of nitrite in feeds most commonly used in livestock diets, no reliable estimates of exposure could be calculated. The highest estimated dietary exposure of cattle to nitrate from feed was for beef cattle fed a grass silage-based diet (53 mg/kg bw per day). For sheep and goats, the categories 'lactating sheep' and 'goats for fattening' had the highest exposure estimates to nitrate from grass silage-based diet, with 46 and 60 mg/kg bw per day, respectively.

In non-ruminants, the exposure estimates are low (from mean upper bound (UB) 0.3 mg/kg bw per day in cats to 5.6 mg/kg bw per day in laying chicken). However, these might be underestimates as a result of lack of data on the main ingredients in their diets.

The risk characterisation of exposure to nitrate is evaluated taking into consideration the comparison between the mean UB exposure estimates and the identified reference points for adverse effects. In ruminants, the $BMDL_{10}$ of 64 mg nitrate/kg bw per day was compared with the highest estimated mean exposures of 53 and 60 mg nitrate/kg bw per day calculated for beef cattle and fattening goats, respectively, when fed grass silage-based diets. This comparison indicates that the exposure may raise a health concern, considering the uncertainty in the high exposure estimates for grass silage and for other forages that may contain relatively high levels of nitrate but for which data are missing.

There are some examples in the literature indicating successful adaptation of the ruminants to nitrate in feed, suggesting that the $BMDL_{10}$ calculated may be conservative. However, due to the large variability in the design and outcome of these studies, it is not possible to set a different reference point for animals which have undergone long-term exposure to elevated levels of nitrate.

Based on the comparison of mean exposure estimates of 2.0 mg nitrate/kg bw per day in starter pigs' feeds with a no observed adverse effect level (NOAEL) of 410 mg nitrate/kg bw per day identified for pigs, their risk of adverse health effects from feeds containing nitrate was considered very low, although the absence of data on certain key ingredients in the diets of this species is likely to have resulted in an underestimation of levels of exposure.

The health risk from the exposure to nitrate in species other than ruminants and pigs and to nitrite in farmed and companion animals could not be assessed due to the limited data available.

There might be formation of toxic N-nitrosamines in feed, and in particular fishmeal, due to the presence of nitrite and secondary amines, although there was no statistical correlation between concentrations of nitrate, nitrite and N-nitrosamines shown in the very old studies available. No recent information is available on N-nitrosamine intoxication of animals, due probably to the setting of maximum limits of nitrite in fishmeal (30 mg/kg). A limited number of old studies with few animal species showed little, if any, formation of N-nitrosamines due to the reaction of nitrite with secondary amines endogenously. However, these studies were made under specific experimental feeding conditions which may be unlikely to be met under commercial feeding practices. The evidence to

assess the risk from the endogenous production of N-nitrosamines is very limited and there is no information to link it with adverse effects in farmed and companion animals.

Based on a limited data set, both the transfer of nitrate and nitrite from feed to food products of animal origin and the nitrate- and nitrite-mediated formation of N-nitrosamines and their transfer into these products are likely to be negligible.

More information is recommended on nitrate and nitrite regarding their toxicokinetics and adverse effects in animal species other than ruminants and pigs, at realistic dietary exposure levels. Occurrence data of nitrate and nitrite in feeds for rabbits, horses, poultry, dogs, cats, fur animals and fish are needed. In addition, collection of occurrence data on nitrate and in particular on nitrite and N-nitrosamines formed due to the presence of nitrate and nitrite in the different major feeds, especially in forages, is recommended in order to produce reliable exposure estimates. More occurrence data of nitrate and nitrite in fresh and ensiled herbages should be sought, e.g. from the annual analysis performed by EU commercial laboratories for livestock farmers, in order to better estimate exposure by ruminant livestock and horses. Finally, more data are needed on the endogenous formation of N-nitrosamines in the different species and on the transfer of nitrate, nitrite and N-nitrosamines, formed due to the presence of nitrate and nitrite in feed, to food products of animal origin.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

BACKGROUND

Maximum levels for nitrite in feed have been established by Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

EFSA adopted a scientific opinion on nitrite as undesirable substance in feed in 2009. EFSA concluded in its Opinion that for pigs and cattle, as representative sensitive food producing species, the margins of safety with respect to the respective no observed adverse effect level (NOAEL) are sufficient. It considered furthermore that the presence of nitrite in animal products does not raise any concern for human health. Directive 2002/32/EC has been amended as regards nitrite in 2010 and 2011 to take into account the outcome of the EFSA Opinion.

A report on European Union controls for nitrite and feed was submitted in 2014 for discussion by the UK delegation to the Standing Committee on Plants, Animals, Feed and Food. The report concluded that 'on the basis of the data reported by EFSA and considered in detail in the review by Cockburn et al. (2013), along with the fact that there has been no evidence of any problems of poisoning from nitrite in feed being reported in UK animal production, it is concluded that there appears to be little evidence to justify maximum levels for nitrite in feed materials. Furthermore, it should be noted that establishing maximum levels for nitrite in feeds does not necessarily protect livestock from poisoning. It is well known that endogenous conversion of dietary nitrate to nitrite occurs, and therefore it is the levels of nitrate in the diet which are likely to have the greatest impact on nitrite exposure. However, there are currently no maximum levels for nitrate in feed.'

End of 2014, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) published an opinion on nitrite and nitrate in feed. It was concluded that 'The maximum levels of nitrite established for feed materials and compound feed can be deleted from EU legislation. Taking into account current knowledge, it is inappropriate to establish maximum levels of nitrate in feed.'

Extensive discussions in the Standing Committee on Plants, Animals, Food and Feed did not result into an unequivocal view on how to proceed as regards the existing provisions on nitrite in feed and the Committee considered that before concluding, it would be necessary to have an EFSA comprehensive Opinion on the risks for animal health related to the presence of nitrite and nitrate in feed. Indeed, endogenous conversion of dietary nitrate into nitrite occurs and it is very likely that the presence of nitrate in feed has the largest impact on nitrite animal exposure. Therefore, it is appropriate to provide for a comprehensive assessment of the risks for animal health related to the animal exposure to nitrite (following presence in feed and endogenous conversion from nitrate into nitrite) and to nitrate itself.

TERMS OF REFERENCE

In accordance with Art. 29 (1) of Regulation (EC) No 178/2002, the European Commission (EC) asks the European Food Safety Authority to provide an opinion on the risks for animal health related to the presence of nitrite and nitrate in feed.

1.2. Interpretation of the Terms of Reference

EFSA issued a scientific opinion in 2009 on nitrite as an undesirable substance in animal feed (EFSA, 2009). The European Commission now asked EFSA to update its previous Opinion in response to reports challenging the appropriateness of setting maximum levels of nitrite in animal feeds. Because of the conversion of nitrate to nitrite in the gastrointestinal tract of animals, the European Commission requests a comprehensive assessment of the risks for animal health related to the exposure to both nitrite and nitrate. The CONTAM Panel assessed nitrate and nitrite as ions. The chemical formulas NO_2^- and NO_3^- are also used for clarity when concentrations or doses are reported.

The CONTAM Panel considered that it would best respond to this mandate by addressing the following questions:

- Are there new data since its last Opinion indicating any additional toxicological effects of nitrate and nitrite, in farmed and companion animals?
- What are the critical effects for each animal species and category and can reference points be identified for these effects?

- Which feed materials used in the EU are the main sources of nitrate and nitrite and what are the levels of nitrate and nitrite in these feeds?
- What are the estimates of exposure to nitrite and nitrate, in feed of different animal species and categories in the European Union?
- What is the estimated risk to animal health due to nitrate and nitrite at the current exposure?
- What are the levels of nitrate and nitrite transfer from feed to food products of animal origin, and would these levels be acceptable?
- What are the levels of the nitrate- and nitrite-mediated formation of N-nitrosamines (in feed and endogenously) and their transfer into food products of animal origin and would these levels be acceptable?

1.3. Additional information

1.3.1. Chemistry, production and use of nitrate and nitrite

Physico-chemical properties of nitrate, nitrite and some selected salts used in food, feed and as fertilisers are depicted in Table 1.

Table 1: Physico-chemical properties of nitrate, nitrite and some selected salts

Parameter	Nitrate	Sodium nitrate	Potassium nitrate	Calcium nitrate (anhydrous)	Calcium nitrate (tetra-hydrate)	Ammonium nitrate	Nitrite	Sodium nitrite	Potassium nitrite
Formula	NO_3^-	$NaNO_3$	KNO_3	$Ca(NO_3)_2$	$Ca(NO_3)_2 \times 4 H_2O$	NH_4NO_3	NO_2^-	$NaNO_2$	KNO_2
CAS Registry number	14797-55-8	7631-99-4	7757-79-1	10124-37-5	13477-34-4	6484-52-2	14797-64-0	7632-00-0	7758-09-0
Molecular mass (g/mol)	62.01	85.00	101.11	164.09	236.15	80.04	46.01	69.00	85.10
Solubility in water (25°C)	Highly soluble	Freely soluble	Freely soluble	1,290 g/L (20°C)		1,877 g/L (20°C)		820 g/L (20°C)	2,810 g/L (20°C)
Melting point (°C)		306.5 (decomposes at 380°C)	334 (decomposes at 400°C)	561	45	169 (decomposes at > 170°C)		270 (decomposes at > 320°C)	441 (decomposition starts at 350°C

Nitrate (NO_3^-) is a polyatomic anion that can form salts with a number of elements of the periodic table. Nitrates naturally occur ubiquitously in the environment, are involved in the nitrogen cycle and build large deposits especially in the form of sodium nitrate ($NaNO_3$) in some regions, e.g. the Atacama-Desert in Chile, thus the trivial name Chile saltpetre for $NaNO_3$. They have various uses in feed and food, such as in the production of fertilisers, and food preservatives. Nitrates are generally highly soluble in water and play a substantial role as nutrients for plants. Thus, they are found in all plants, especially in green leafy vegetables. Regarding feed and food, sodium nitrate and potassium nitrate are of special importance.

Sodium nitrate is a white crystalline, slightly hygroscopic powder. Potassium nitrate is a white crystalline powder or transparent prisms having a cooling, saline, pungent taste. Sodium nitrate (E 251) and potassium nitrate (E 252) are authorised food additives in 24 food categories in the European Union in line with the Annex II of Regulation (EC) No 1333/2008[1] . They are commonly used as preservatives and combined with nitrite salts in curing mixtures (i.e. sodium chloride solutions) for meats to develop and fix the colour of meat, to inhibit microbial growth and to develop characteristic flavours (EFSA ANS Panel, 2017b). In veterinary medicine, potassium nitrate is used as diuretic in pigs, cattle and horses.

Two other nitrate that are used especially in fertilisers are calcium nitrate ($Ca(NO_3)_2$) and ammonium nitrate (NH_4NO_3). Anhydrous $Ca(NO_3)_2$ is colourless, hygroscopic and thus absorbs easily moisture and forms tetrahydrates. NH_4NO_3 is a colourless crystalline salt

Nitrite (NO_2^-) is the anion of inorganic nitrite salts. Natural occurrence of nitrite in the environment is a consequence of the nitrogen cycle, but usually nitrite is found in very low concentration. Nitrite is formed in nature by the action of nitrifying bacteria as an intermediate stage in the formation of nitrate. Synthetically, nitrites of the alkali earth metals can be produced by reacting a mixture of nitrogen monoxide (NO) and nitrogen dioxide (NO_2) with the corresponding metal hydroxide solution, as well as through the thermal decomposition of the corresponding nitrate. Nitrite can be reduced to nitric oxide or ammonia by many species of bacteria. The most important nitrites in feed and food are sodium nitrite ($NaNO_2$) and potassium nitrite (KNO_2).

Sodium nitrite is a white to slightly yellowish crystalline powder. It is hygroscopic, has a melting point of 270°C and decomposes above 320°C. It slowly oxidises in the air to sodium nitrate. It is used in various applications and the manufacturing of numerous compounds. In human and veterinary medicine, $NaNO_2$ has been used as a vasodilator, bronchodilator and as an antidote for cyanide poisoning. In veterinary medicine, the substance is intended for use (together with other antimicrobial agents or biocides) as an antiseptic by topical application to the teats of dairy cows after milking in order to prevent mastitis. $NaNO_2$ is authorised as food additive E 250 in line with Annex II of Regulation (EC) No 1333/2008. As a food additive, it stabilises the colour of preserved fish and meats and also inhibits the growth of *Clostridium botulinum*, the bacterium, which produces the botulinum toxin.

Potassium nitrite (KNO_2) is a white to slightly yellow crystalline powder. KNO_2 is an authorised food additive coded E 249 in line with the Annex II of Regulation (EC) No 1333/2008. It is used as a colour fixative in fish products and in pickling and curing meat, sometimes in combination with sodium nitrite and with potassium and sodium nitrate. Like sodium nitrite, it inhibits the growth of the botulism-causing bacterium *Clostridium botulinum*.

When reacting with secondary amines under acidic conditions, nitrite can form N-nitrosamines. This reaction could be potentially of toxicological relevance because some of the dialkyl- or cyclic N-nitrosamines are genotoxic and carcinogenic. Besides the exogenous exposure via food or feed, an endogenous formation has also been reported (EFSA ANS Panel, 2017a) (see Section 3.1.3.3).

1.3.2. Methods of analysis

Most of the methods applied to determine nitrate and nitrite make use of spectrophotometric measurements, often with conversion of nitrate to nitrite or vice versa. The international standardisation bodies European Committee for Standardization (CEN) and International Organisation for Standardisation (ISO) have approved several analytical methods for the determination of nitrate/ nitrite in meat, milk products and vegetables. The two methods approved for meat products make use of spectrometric determination at a wavelength of 540 nm, and ultraviolet detection at 205 nm after

[1] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.

extraction and clean-up (CEN, 2005a,b). The limit of detection (LOD) for the latter method is given as 10 mg/kg. The spectrometric method involves a reduction step whereby nitrate is reduced to nitrite. Without this reduction step, the method can also be applied successfully for the determination of nitrite.

ISO describes three alternative methods for the determination of nitrate and nitrite in milk and milk products (ISO, 2004a,b,c). All include a reduction of nitrate to nitrite, which is measured spectrometrically. Thus, both anions can be determined, the content of nitrate is calculated as the difference between reduced and non-reduced sample extract.

A CEN method describes the enzymatic determination of nitrate in vegetable-containing food for babies and infants. The method is applicable to nitrate concentrations in the range of 50–200 mg/kg (CEN, 1997). In 2017, CEN published a high-performance liquid chromatographic (HPLC)/ion chromatographic (IC) method for the determination of nitrate levels in vegetables and vegetable products with a limit of quantification (LOQ) of 25 mg/kg (CEN, 2017).

The Nordic Committee of Analysis of Food (NMKL, 2013) specifies a spectrophotometric method for the determination of nitrate/nitrite content in foodstuffs and water after zinc reduction and very sensitive and widely quantification using Griess reaction. The method has been validated in vegetables (lettuce), meat products, baby food, milk and surface water. The LOD of nitrate for meat products is 5 mg/kg.

The Official Methods of Analysis of AOAC INTERNATIONAL present two photometric methods for the determination of nitrate/nitrite in meat and cured meat (AOAC, 2005). The method based on the Griess reaction with a measurement at 540 nm was adopted as a Codex Reference method (Type II) for nitrite and potassium and/or sodium salts in canned corned beef and luncheon meat.

More recent methods make use of ion chromatography with conductivity detection enabling LODs and LOQs of 4 and 10 mg/kg, respectively, in vegetables (Chung et al., 2011). Iammarino et al. (2013) reported on the determination of nitrate and nitrite using an in-house validated ion chromatographic method with electrochemical detection. The method was applied to the analysis of 1,785 samples of fresh meat products, shellfishes, diary product and leafy vegetables. The LOD for nitrate was 3.2 mg/kg. Croitoru (2012) developed a high-performance liquid chromatography (HPLC)-ultraviolet (UV)/visible (VIS) method for the determination of low concentrations of nitrite and nitrate in vegetables and biological samples. The method combines the simultaneous VIS detection of the nitrite-related azo dye (Griess reaction), with the simple UV detection of nitrate.

Wang et al. (2017) reviewed the analytical methods that have been published since 2000 and described the detection principles, analytical parameters and advantages and disadvantages. They concluded that, in comparison to other methods, spectrofluorimetric methods have become more attractive due to their availability, high sensitivity and selectivity, low limits of detection and low cost.

1.3.3. Previous assessments

1.3.3.1. In food

Nitrate and nitrite were reviewed by the Scientific Committee on Food (SCF, 1992, 1997) and the Joint Food and Agriculture Organization/World Health Organization (WHO) Expert Committee on Food Additives on several occasions (JECFA, 1962, 1965, 1974, 1976, 1995, 1996, 2002, 2003a,b). The acceptable daily intake (ADI) set by the SCF and the Joint Food and Agriculture Organization/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) for sodium and potassium nitrate (expressed as the nitrate ion) is 0–3.7 mg/kg body weight (bw) per day (SCF, 1997; JECFA, 2003b). The ADI for sodium and potassium nitrite (expressed as nitrite ion) established by the SCF in 1997 is 0–0.06 mg/kg bw per day. The ADI for sodium and potassium nitrite (expressed as nitrite ion) established by JECFA in 2002 is 0–0.07 mg/kg bw per day.

A risk assessment of the intake of naturally occurring nitrate and its metabolites from vegetables with respect to the risks and benefits of exposure to nitrate from vegetables has been performed by the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM). Dietary exposure estimates showed that the ADI for nitrate would not be exceeded by an adult eating 400 g of mixed vegetables. However, high level consumers of vegetables grown under unfavourable local production conditions may exceed the ADI approximately twofold. The CONTAM Panel concluded that the beneficial effects of consumption of vegetables prevail to health risks (EFSA, 2008).

The CONTAM Panel also delivered a statement on the potential health risks for infants and young children from the presence of nitrate in leafy vegetables (EFSA CONTAM Panel, 2010). The CONTAM Panel concluded that levels of nitrate in lettuce are not a health concern, but the concentrations of

nitrate in spinach have the potential to increase dietary nitrate exposure to levels at which a health concern cannot be excluded for some young children. Inappropriate storage of cooked vegetables, especially of spinach can result in direct conversion of nitrate to nitrite, resulting in greatly increased potential for causing methaemoglobinaemia.

In 2017, the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS Panel) issued an opinion on the safety of nitrites as food additives (EFSA ANS Panel, 2017a). The Panel concluded that an increased MetHb level, observed in human and animals, was a relevant effect for the derivation of the ADI. The Panel, using a BMD approach, derived an ADI of 0.07 mg nitrite/kg bw per day. The exposure to nitrite resulting from its use as food additive did not exceed this ADI for the general population, except for a slight exceedance in children at the highest percentile. The Panel found no concern from the endogenous formation of nitrosamines from nitrite. There was health risk identified for nitrosamines found in meat products; however, it was not possible to clearly discern nitrosamines produced from the nitrite added at the authorised levels, from those found in the food matrix without addition of external nitrite.

In 2017, EFSA's ANS Panel also issued an opinion on the safety of nitrate as food additives (EFSA ANS Panel, 2017b). The Panel considered the derivation of an ADI for nitrate from the ADI of nitrite, based on the formation of methaemoglobin (MetHb, expressed as percentage of total haemoglobin), following the conversion of salivary nitrate to nitrite. However, there were large variations in the data on the nitrate-to-nitrite conversion in the saliva in humans, and therefore, the derivation of a single ADI value from the available data was not possible. The Panel noticed that even using the highest nitrate-to-nitrite conversion factor, the MetHb levels produced due to nitrite obtained from this conversion would not be clinically significant. In addition, the theoretically estimated production of endogenous N-nitroso compounds would be of low concern. The Panel concluded that there was insufficient evidence to withdraw the ADI established by SCF. The exposure to nitrate solely from its use as a food additive was estimated to be less than 5% of the overall exposure to nitrate in food based on a refined estimated exposure scenario.

The International Agency for Research on Cancer (IARC) reviewed and evaluated the effects of ingested nitrate and nitrite in experimental animals and in humans (IARC, 2010). Concerning the human data, IARC concluded that there was insufficient evidence to support that nitrate is carcinogenic. However, nitrosating agents produced from nitrite under acidic conditions in the stomach could react readily with nitrosatable compounds, especially secondary amines and amides, to generate N-nitroso compounds, some of which are known carcinogens. Taken into consideration these aspects, the IARC further concluded that under conditions that result in endogenous nitrosation and enhancing the production of N-nitroso compounds, ingested nitrate or nitrite are probably carcinogenic to humans (Group 2A).

In 2017, the WHO Guidelines for Drinking-water Quality[2] set the guideline value for nitrate in water at 50 mg/L, and for nitrite at 3 mg/L in order to protect the most sensitive subpopulation, bottle-fed infants, against methaemoglobinaemia (nitrate and nitrite) and effects on the thyroid (nitrate). A Hazard Index of 1 should not be exceeded for combined exposure to nitrate and nitrite.

Health Canada has published a report on the metabolite N-nitrosodimethylamine (NDMA) (Health Canada, 2011), as well as a document on nitrate and nitrite guidelines in drinking water (Health Canada, 2013). The maximum acceptable concentration for NDMA in drinking water is 0.04 μg/L. The maximum acceptable concentration for nitrate has been established at 45 mg/L; the drinking water guideline for nitrite stipulated a maximum acceptable concentration of 3 mg/L.

The Australian Food Safety Authority has published a report on nitrate and nitrite in 2011 (FSANZ, 2011) estimating that the Australian dietary nitrate and nitrite exposures are not considered to represent an appreciable health and safety risk.

1.3.3.2. In feed

In 2009, EFSA's CONTAM Panel provided an opinion on nitrite as undesirable substance in animal feed. The Panel calculated margins of exposure, comparing the estimated nitrite intakes for pigs and cattle and the respective NOAELs and considered that these do not pose concerns for animal health given that livestock are husbanded under good agricultural practices.

[2] https://apps.who.int/iris/bitstream/handle/10665/254637/9789241549950-eng.pdf?sequence=1

In 2014, ANSES (ANSES, 2014) published an opinion on the possible modification of the Directive 2002/32/EC[3] on substances undesirable in animal feed in relation to nitrite and nitrate. The opinion assessed the potential risks for humans, animals and the environment in the case the maximum permitted levels of nitrite in raw feed materials would be deleted while levels for complete feed would remain. The opinion concluded that both maximum levels of nitrite in raw material and complete feed for animals can be deleted and that, due to the limited data, it would not be appropriate to set maximum limits for nitrate.

1.3.4. Legislation

In this Opinion, where reference is made to European legislation, the reference should be understood as relating to the most recent amendment at the time of publication of this Opinion, unless otherwise stated.

1.3.4.1. Feed

According to Article 3 of Directive 2002/32/EC of the European Parliament and of the Council *'products intended for animal feed may enter for use in the Community from third countries, be put into circulation and/or used in the Community only if they are sound, genuine and of merchantable quality and therefore when correctly used do not represent any danger to human health, animal health or to the environment or could adversely affect livestock production.'* In particular, products intended for animal feed shall be deemed not to be in conformity with legislation if the level of undesirable substances they contain does not comply with the maximum levels laid down in Annex I of the Directive. The maximum contents for sodium nitrite in certain feed products as laid down in Annex I of Directive 2002/32/EC are shown in Table 2.

Table 2: EU legislation on sodium nitrite in feed materials as listed currently in Annex I of Directive 2002/32/EC

Undesirable substance	Products intended for animal feed	Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12%
Nitrite (*The maximum levels are expressed as sodium nitrite*)	Feed materials	15
	with the exception of:	
	— fishmeal;	30
	— silage;	—
	— products and by-products from sugar beet and sugarcane and from starch and alcoholic drink production	—
	Complete feed	15
	with the exception of:	
	— complete feed for dogs and cats with a moisture content exceeding 20%	—

The sodium nitrite levels described in Table 2 correspond to a maximum content of the nitrite ion of 20 mg/kg in fishmeal and 10 mg/kg in the respective feedingstuffs where maximum contents are set.

Nitrite is not only regulated as an undesirable substance in feed but also listed in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003[4] as an authorised preservative as sodium nitrite E 250 in canned dog and cat feed up to a maximum level of 100 mg/kg, and as a silage additive.[5]

For nitrate, no maximum contents in feed materials are set in Directive 2002/32/EC.

[3] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 30.5.2002, p. 10–22.
[4] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29–43.
[5] https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed-eu-reg-comm_register_feed_additives_1831-03.pdf

1.3.4.2. Water for drinking

Harmonised maximum contents for nitrate and nitrite in water for drinking by animals are not stipulated. However, Annex III of Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene[6] requires that '*Water for drinking or for aquaculture shall be of appropriate quality for the animals being produced. Where there is cause for concern about contamination of animals or animal products from the water, measures shall be taken to evaluate and minimize the hazards. Feeding and watering equipment must be designed, constructed and placed in such a way that contamination of feed and water is minimized. Watering systems shall be cleaned and maintained regularly, where possible.*'

The German authorities have set a number of orientation values for the appraisal of drinking water for animals in the context of feed and food safety. For nitrate, orientation values of < 300 mg/L for ruminant animals and < 200 mg/L for calves and other animal species are suggested.[7] Orientation values of < 30 mg/L were recommended for nitrite.

Regarding nitrate, international efforts have been put in place to reduce and limit its occurrence in water using Good Agricultural Practice (GAP) controlling the application of nitrogen fertiliser and/or manures limiting concentrations of inorganic nitrogenous compounds in ground and surface waters. Council Directive 91/676/EEC[8] concerning the protection of waters against pollution caused by nitrate from agricultural sources has been implemented to protect waters from nitrate pollution in EU countries from agricultural sources.

1.3.4.3. Food

In line with Article 9 of Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs[9], Member States shall monitor nitrate levels in vegetables which may contain significant levels, in particular green leafy vegetables, and communicate the results to EFSA on a regular basis. The maximum levels (MLs) currently set by Regulation (EC) No 1881/2006 are presented in Table 3. All MLs apply to the edible part of the foodstuffs concerned. It should be noted that the MLs for fresh lettuce and rucola vary depending on the time of harvesting. Moreover, the growing conditions, whether under cover or in the open air, have an influence on the MLs for fresh lettuce and Iceberg type lettuce.

Table 3: Maximum levels for nitrate in food as listed currently in the Annex, Section 1 of Regulation (EC) No 1881/2006

Foodstuffs		Maximum levels (mg NO_3^-/kg)	
1.1	Fresh spinach (*Spinacia oleracea*)[(a)]		3,500
1.2	Preserved, deep frozen or frozen spinach		2,000
1.3	Fresh Lettuce (*Lactuca sativa* L.) (protected and open-grown lettuce) excluding lettuce listed in point 1.4	Harvested 1 October to 31 March:	
		Lettuce grown under cover	5,000
		Lettuce grown in the open air	4,000
		Harvested 1 April to 30 September:	
		Lettuce grown under cover	4,000
		Lettuce grown in the open air	3,000
1.4	'Iceberg' type lettuce	Lettuce grown under cover	2,500
		Lettuce grown in the open air	2,000
1.5	Rucola (*Eruca sativa*, *Diplotaxis* sp., *Brassica tenuifolia*, *Sisymbrium tenuifolium*)	Harvested 1 October to 31 March:	7,000
		Harvested 1 April to 30 September:	6,000
1.6	Processed cereal-based foods and baby foods for infants and young children[(b),(c)]		200

[6] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1–22.
[7] www.bmel.de; Hygienische Qualität von Tränkwasser - Orientierungsrahmen zur futtermittelrechtlichen Beurteilung
[8] OJ L 375, 31.12.1991, p. 1–8.
[9] OJ L 364, 20.12.2006, p. 5–24.

(a): The maximum levels do not apply for fresh spinach to be subjected to processing and which is directly transported in bulk from field to processing plant.
(b): Foodstuffs listed in this category as defined in Regulation (EU) No 609/2013[10] of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/ EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009.
(c): The maximum level refers to the products ready to use (marketed as such or after reconstitution as instructed by the manufacturer).

Potassium and sodium nitrite (E 249/E 250) and sodium and potassium nitrate (E 251/E 252) are authorised food additives in line with Annex II of Regulation (EC) No 1333/2008 on food additives up to 180 mg/kg of product and 500 mg/kg of product, respectively.

Maximum levels (termed 'parametric values') for nitrite of 0.5 mg/L and nitrate of 50 mg/L in drinking water intended for human consumption are laid down in Council Directive 98/83/EC[11]. The Directive stipulates that Member States must ensure that the condition that [nitrate]/50 + [nitrite]/3 = ≤ 1, is complied with and that the value of 0.10 mg/L for nitrite is complied with ex water treatment works. The square brackets signify the concentrations in mg/L for nitrate and nitrite.

1.3.4.4. Medicinal products

In 1997, the Committee for Veterinary Medicinal Products of the European Agency for the Evaluation of Medical Products (EMEA)[12] evaluated the use of potassium nitrate as diuretic in pigs, cattle and horses where it is administered by the oral route at doses up to 30 g per animal per day. The Committee concluded that there is no need to establish a maximum residue limit (MRL) for potassium nitrate and recommended its inclusion into Annex II of Council Regulation (EEC) No 2377/90[13]. In 2006, the Committee for Veterinary Medicinal Products of the European Agency for the Evaluation of Medical Products (EMEA)[14] evaluated the use of sodium nitrite as a disinfectant by topical application to the teats of dairy cows after milking in order to prevent mastitis. The Committee concluded that there is no need to establish a maximum residue limit (MRL) for sodium nitrite and recommended its inclusion into Annex II of Council Regulation (EEC) No 2377/90. This Regulation is no longer in force and was repealed by Regulation (EC) 470/2009[15]. The list of pharmaceutical active compounds in Annex II of Council Regulation (EEC) No 2377/90 can now be found in the Annex, Table 1 of Commission Regulation (EU) No 37/2010[16] on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. The entry for potassium nitrate lays down that it can be used for all food producing animals and no MRL is required. The entry for sodium nitrite stipulates that it can only be applied for topical use in bovine and no MRL is required.

2. Data and methodologies

2.1. Data

2.1.1. Data on hazard identification and characterisation

Data were obtained from the scientific literature. Additionally, raw data of relevant studies were requested to authors for further evaluation (see Section 6. Documentation as provided to EFSA).

[10] OJ L 181, 29.6.2013, p. 35–56.
[11] Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. OJ L 330, 5.12.1998, p. 32–54.
[12] EMEA/MRL/232/97-FINAL, July 1997.
[13] Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ L 224, 18.8.90, p. 1–8.
[14] EMEA/CVMP/116350/2006-FINAL, April 2006.
[15] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council. OJ L 152, 16.6.2009, p. 11–22.
[16] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1–72.

2.1.2. Occurrence data submitted to EFSA

2.1.2.1. Data collection and validation

Following an European Commission mandate to EFSA, a call for annual collection of chemical contaminant occurrence data in feed, including nitrate and nitrite, was issued by the former EFSA Dietary and Chemical Monitoring Unit (now DATA Unit)[17] in December 2010.[18] European national authorities and similar bodies, research institutions, academia, food business operators and other stakeholders were invited to submit analytical data on nitrate and nitrite in feed.

The data submission to EFSA followed the requirements of the EFSA Guidance on Standard Sample Description for Food and Feed (EFSA, 2010a); occurrence data were managed following the EFSA standard operational procedures (SOPs) on 'Data collection and validation' and on 'Data analysis of food consumption and occurrence data'.

Data on nitrate and nitrite in feed submitted to EFSA by the end of October 2019 were considered for the present assessment. Data received after that date were not included.

Additional information on nitrate levels in fresh grass and grass silage has been obtained as a personal communication (see Section 3.2.2).

2.1.2.2. Data analysis

Following EFSA's SOP on 'Data analysis of food consumption and occurrence data' to guarantee an appropriate quality of the data used in the exposure assessment, the initial data set was carefully evaluated by applying several data cleaning and validation steps. Special attention was paid to identification of duplicates and to accuracy of different parameters such as 'Sampling country', 'Sampling year', 'Sampling strategy', 'Analytical methods', 'Result express', 'Reporting unit', 'Limit of detection/quantification' and the codification of analytical results under FoodEx classification (EFSA, 2011a). The outcome of the data analysis is presented in Section 3.2.2.

The left-censored data (LCD) (results below LOD or below LOQ) were treated by the substitution method as recommended in the 'Principles and Methods for the Risk Assessment of Chemicals in Food' (WHO/IPCS, 2009). The same method is indicated in the EFSA scientific report 'Management of left-censored data in dietary exposure assessment of chemical substances' (EFSA, 2010b) as an option in the treatment of left-censored data. The guidance suggests that the lower bound (LB) and upper bound (UB) approach should be used for chemicals likely to be present in the food (e.g. naturally occurring contaminants, nutrients and mycotoxins). The LB is obtained by assigning a value of zero (minimum possible value) to all samples reported as lower than the LOD (< LOD) or LOQ (< LOQ). The UB is obtained by assigning the numerical value of LOD to values reported as < LOD and LOQ to values reported as < LOQ (maximum possible value), depending on whether LOD or LOQ is reported by the laboratory.

Less than 1% of the analytical results were reported to EFSA as corrected for recovery and approximately 8% of analytical results were reported as not corrected for recovery, whilst for the remaining part of data, the information on recovery was not provided. When recovery rates are not reported, the analytical results submitted cannot be corrected. It is expected that less than 100% of the analyte concentration of these samples is recovered, and therefore, the analytical results reported are generally lower than the 'real' ones.

2.1.3. Feed consumption data

No comprehensive feed consumption database exists in the EU, and therefore, the types and amounts of feeds consumed by the most relevant farmed livestock and companion animals have been based on estimates, details of which are given in Section 2.2.2 and Annex II.

2.1.4. Feed classification

Feed samples were classified according to the Catalogue of feed materials as described in Commission Regulation No 68/2013[19] and transposed to the FoodEx classification system of EFSA.

[17] From 1 January 2014 onwards, Evidence Management Unit (DATA).
[18] http://www.efsa.europa.eu/en/consultations/call/190410
[19] Commission Regulation (EU) No 68/2013 of 16 January 2013 on the Catalogue of feed materials. OJ L 29, 30.1.2013, p. 1–64.

2.2. Methodologies

2.2.1. Methodology for data collection from the literature and study appraisal

The data from literature were collected as described in Annex I. The information retrieved was screened and evaluated by relevant domain experts from the CONTAM Working Group on nitrate and nitrite in feed and used for the present assessment. Limitations in the information used are documented in this Scientific Opinion. The selection of the scientific papers for inclusion or exclusion was based on consideration of the extent to which the study was relevant to the assessment or on general study quality considerations (e.g. sufficient details on the methodology, performance and outcome of the study, on dosing, substance studied and route of administration and on statistical description of the results), irrespective of the results.

In experimental studies, the doses provided as mg/kg of dry matter (DM) of feed/diet were transformed in mg/kg animal bw per day to facilitate comparison among studies according to the formula:

$$\text{Dose} = \text{mg/kg DM} \times \text{Dry Matter Intake (DMI)/average body weight (bw)}$$

For this calculation, data on the average bw and DMI of the animals are needed, but not always provided in the publications. If not provided, values reported in the EFSA scientific report on the animal dietary exposure (EFSA, 2019) were used. Exceptionally, for Jersey cow, default values from NRC (2001) were used as their body weight is known to be lower than other lactating cows' breeds. In studies with growing animals, the average body weight was calculated using the formula:

$$\text{Average bw} = (\text{initial bw} + \text{final bw})/2$$

In studies where nitrate was dosed in water but water intake was not provided, the water intake (WI) has been calculated as 3x DMI in line with EFSA FEEDAP Panel suggestion (EFSA FEEDAP Panel, 2018).

2.2.2. Methodologies for dietary exposure assessment in animals

No comprehensive feed consumption database exists in the EU. Therefore, assessment of dietary exposure has been based on assumptions on the types of feed, and the amounts, consumed by farm and companion animals.

Compound feeds may account for a large proportion – and in some cases all – of the diet of farmed livestock and companion animals, but no data on levels of nitrate or nitrite in compound or complementary feeds were available. Therefore, assessment of dietary exposure has been based on assumptions on the types of feed materials, and the amounts, consumed. For ruminants and horses, forages – fed either fresh or conserved (e.g. as hay or silage) – usually represent the major component of the diet and are supplemented with individual feed materials or compound feeds.

Data on nitrate and nitrite contents of feeds used to estimate exposure are given in Tables 6 and 7, respectively. From these data, it is clear that there are major gaps in the database, particularly for nitrite, for the most commonly used livestock feeds.

For nitrate, and in the absence of any data on levels in compound feeds, levels of nitrate in individual feeds available on the EFSA database (Annex III) have been used together with example diets (see Annex II), to estimate exposure. A wide range of livestock production and feeding regimes are employed in the EU, and it is beyond the scope of this Opinion to attempt to encompass all the variables involved. It should be stressed therefore that the example diets used here do not represent either 'average' or 'extreme' diets, nor are the feeding systems 'typical' for all of Europe. Instead, the diets are used to estimate levels of exposure that might be indicative. They are based on published guidelines on nutrition and feeding (AFRC, 1993; Carabaño and Piquer, 1998; NRC, 2007a,b; Leeson and Summers, 2008; McDonald et al., 2011; EFSA FEEDAP Panel, 2012; OECD, 2013), and expert knowledge of production systems in Europe. Details of the rations used, feed intakes and live weights assumed are given in Annex II.

According to EFSA (2011b), caution is needed when calculating acute exposure (95th percentile) where data on less than 60 samples are available, since the results may not be statistically robust. Therefore, in view of the limited database the lack of data, only mean exposures have been estimated based on the mean LB and UB concentrations.

Forages are essential ingredients in the diets of ruminants and horses, and a wide range of forage crops are used. They may be fed fresh or conserved, in the case of the latter as dry feeds (e.g. hay)

or ensiled as silage. However, as discussed below (Sections 3.2.1 and 3.4.3.1), the only reliable data available to estimate exposure in this Opinion were for grass silage and maize silage.

2.2.3. Methodology applied for risk assessment

The CONTAM Panel applied the general principles of the risk assessment process for chemicals which include hazard identification and characterisation, exposure assessment and risk characterisation. EFSA guidances pertaining to risk assessment have been applied for the present assessment. For a list of the specific EFSA guidance applied, see Annex IV.

3. Assessment

3.1. Hazard identification and characterisation

3.1.1. Toxicokinetics

The kinetics of nitrate and nitrite are strictly inter-related so that they will be treated together. Few data on the fate of nitrate and nitrite in animals have been reported after the publication of the Opinion on nitrite as undesirable substances in animal feed in 2009 (EFSA, 2009). The main concepts will be summarised below, while the species-related information will be dealt with separately.

In livestock ruminant and monogastric species (pigs, horses), the oral absorption of nitrate and nitrite is low (10–20%) and occurs mainly in the rumen/stomach and upper intestine.

Nitrate is easily reduced to nitrite. In the rumen, the microbiota performs the further reduction of nitrite to ammonia, which is then utilised by microorganisms for amino acid synthesis or eliminated through eructation. In case of high intake, the latter pathway is saturated resulting in increased amounts of nitrite being absorbed though the ruminal wall and reaching the bloodstream, with extensive MetHb formation. In monogastric species like horses and pigs, which are unable to convert nitrite to ammonia, nitrate reduction takes place mostly in the distal tract of the intestine, leading therefore to a lower absorption rate of the formed nitrite. In addition, nitrate is actively secreted in the oral cavity in certain monogastric species (humans, dogs, pigs) and then reduced to nitrite by bacterial nitroreductases mainly found in the oral cavity (EFSA, 2009; EFSA ANS Panel, 2017b).

Once absorbed, nitrite is rapidly distributed via the bloodstream to tissues. After the i.v. administration of sodium nitrite (20 mg/kg bw), wide interspecies differences in the volume of distribution (Vd) have been recorded between dogs (1,624 mL/kg, sheep (278 mL/kg) and horses (ponies) (192 mL/kg) (Schneider and Yeary, 1975; EFSA, 2009). The available data (*in vitro* study in dogs) point to a limited to negligible binding of nitrite (up to around 14%) and nitrate (< 1%) to plasma proteins at equimolar concentrations. Nitrite is partly taken up by erythrocytes and known to bind tightly to the haem iron of Hb, forming MetHb and nitric oxide ($NO^{\bullet}$, see below).

Under the acidic conditions of the stomach, nitrite is rapidly converted to nitrous acid, a highly unstable compound which spontaneously decomposes to various nitrogen oxides, including nitric oxide ($NO^{\bullet}$), that is implicated in a variety of physio-pathological effects acting as a second messenger (Bryan and Lancaster et al., 2017; Hancock and Neill, 2019). $NO^{\bullet}$ may also be formed physiologically at much lower levels through the urea cycle using L-arginine as the substrate and NO-synthase. A further metabolic pathway involving nitrite is the oxidation to nitrate, which in rats occurs in liver, heart and other tissues and is accomplished by cytochrome c oxidase, myoglobin and cytochrome P450 (Curtis et al., 2012). Another major source of nitrate is the nitrite-mediated oxidation of oxyhaemoglobin (HbO_2) to MetHb according to the following stoichiometry: $4HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb^+(MetHb) + 4NO_3^- + O_2 + H_2O$ (Kosaka and Tyuma, 1987).

The extensive oxidation of nitrite to nitrate also takes place in target species. In the quoted study by Schneider and Yeary (1975), the i.v. injection of 20 mg sodium nitrite/kg bw to dogs, sheep and ponies, resulted in blood nitrate levels of the same order of magnitude with calculated Vd of 239, 291 and 209 mL/min, respectively.

Nitrate and nitrite are mainly eliminated via the urinary route. Based on experimental studies performed in dogs, sheep and ponies, the biological half-lives of nitrite are at least one order of magnitude shorter (around 0.5 h) than those of nitrate (range 4–44 h). Nitrite is indeed rapidly and extensively excreted in urine, while a large fraction of primary urinary nitrate (approx. 80%) undergoes a reabsorption, which is likely mediated by an active transport process (Qin et al., 2012).

Nitrate excretion in milk has been reported in ruminant species (EFSA, 2009; Jones et al., 2013).

3.1.1.1. Species-specific kinetics

Ruminants (cattle, sheep, goat)

Ruminal metabolism

Nitrate and nitrite ruminal metabolism has been recently reviewed (Latham et al., 2016; Nolan, 2016). The main ruminal metabolic pathway involves a two-step reduction of nitrate first to nitrite and then mainly to ammonia, which represents an important nitrogen source for bacterial protein synthesis (Jones, 1972). Nitric- or nitrous oxides may also be formed; however, although conflicting results are reported in the literature, it is generally accepted that the so-called 'denitrification' process ultimately leading to the formation of $NO^{\bullet}$ and N_2O is of minor importance in the overall ruminal fate of nitrate and nitrite (Latham et al., 2016).

The reductive pathway is mainly accomplished by few bacterial species (e.g. *Selenomonas ruminantium*, *Veillonella parvula*, *Wolinella succinogenes*, *Ruminococcus albus, Ruminococcus flavefaciens*) (Lee et al., 2017). *In vitro* experiments with cattle rumen fluid suggest that also protozoa provide a significant contribution (Lin et al., 2011) although their *in vivo* overall role in nitrate reduction remains to be established (Latham et al., 2016). The enzymes mainly involved in the reductive processes are bacterial reductases encoded by different genes, NADH and FADH acting as the reducing equivalents (Moreno-Vivián et al., 1999). Of note, in the rumen, the electron flow is mostly directed to CO_2 acting as a terminal acceptor to generate methane (CH_4), which is the main mechanism to dispose excess H_2 produced during fermentation. CH_4 represents one of the most important greenhouse gases (reviewed by Yang et al., 2016). CH_4 production also causes a loss of 2–12% of gross feed energy to the animal. Based on a considerable wealth of knowledge, it may be concluded that nitrate/nitrite successfully compete with CO_2 as terminal electron acceptors and are nowadays considered of interest as dietary supplements to reduce environmental and economic costs linked to methane emission (Lee and Beauchemin, 2014; Latham et al., 2016; Granja-Salcedo et al., 2019).

Nitrate and nitrite adaptation

It is known that the abrupt exposure of non-adapted microbial ruminal populations to high nitrate dietary intake triggers a rapid induction of nitrate reducing activity and generates large amounts of nitrite. This exceeds the capacity for reduction, partly because nitrite reduction takes place at a slower rate compared to nitrate reduction (Allison and Reddy et al., 1984; Lin et al., 2013). The resulting nitrite are therefore free to enter the bloodstream and cause the formation of MetHb and other adverse effects leading to acute or chronic forms of nitrate toxicosis (Bruning-Fann and Kaneene, 1993).

Conversely, the stepwise increase of nitrate dietary intake is reported to cause significant changes in the composition and activity of microbial populations leading to an increased concentration of nitrate-reducing microorganisms as demonstrated by several *in vitro* investigations (reviewed by Latham et al., 2016). Conflicting results were reported for nitrite-reducing microorganisms. Rumen fluid was collected from steers progressively adapted to increasing nitrate (NO_3^--N) or ammonia (NH_4-N) for 15 days and incubated with sodium nitrate 7.7 mM. While the rate of nitrate disappearance was higher in adapted steers (NO_3^--N) with respect to non-adapted ones (NH_4^+-N), nitrite accumulation was the same in ruminal fluid from NO_3^--N or NH_4^+-N adapted steers (Lin et al., 2013). More recently, rumen fluid from untreated sheep or from sheep administered with daily doses of 6 or 9 g potassium nitrate for 3 weeks showed a dose-related increase in both nitrate and nitrite reductase activity up to approximately five- and threefold, respectively. Both effects were attributed to the treatment-related increase in the abundance of *Selenomonas ruminantium* (Asanuma et al., 2015).

On the whole, the adaptation process is believed to make ruminants less sensitive to the adverse effects of nitrate intake (Nolan, 2016). Alaboudi and Jones (1985) studied the adaptation to nitrate in sheep. In a first trial (exp 1), four rumen-fistulated Dorset-Columbia crossbred ewes (50–60 kg) were fed with a basal diet containing 0.5 g of NO_3^--N/kg DM for 3 weeks and then supplemented with increasing dosages of potassium nitrate (0.5 g/kg bw per day) at 2-week intervals (0.5–1.0–1.5–2.0–2.5 g/kg bw per day) for further 8 weeks. At the beginning of the supplementation (T = 0) and at week 2, 4, 6 and 8, ruminal fluid samples were checked for their *in vitro* nitrate- and nitrite-reducing capacity. The KNO_3 supplementation was then discontinued and animals were monitored again 3 weeks after treatment cessation. As compared to T = 0, there was a progressive increase in both rumen nitrate- (up to threefold) and nitrite-reducing capacity (up to fivefold), with a return to the basal levels (T = 0) 3 weeks after treatment withdrawal. A second trial (exp 2) involved one control

(basal diet, 0.05 g of NO_3^-/kg bw per day) and one adapted sheep (basal diet 0.9 g NO_3^-/kg bw per day); the latter displayed a more than threefold concentration in 'nitrate reducing bacteria'. After 2 weeks, ruminal and blood samples were taken 60 min before (T = –60) and at 30-min intervals (up to 150 min) after nitrate dosing of the adapted sheep, respectively. Peak levels of nitrate and nitrite were reached 90 min after dosing with rapid decline to pre-dosing levels at 150 min. MetHb levels (expressed as percentage of total haemoglobin) were very low (0–0.4%) in the control sheep at all time points and at T = –60 in the treated sheep. In this animal, there was only a modest increase (up to 1.8 %) upon blood nitrate and nitrite peak. The authors concluded that sheep are quite resistant to nitrate toxicity compared to other ruminants and that dietary adaptation further improves the tolerance to nitrate.

Data on cattle are mainly related to the use of nitrate for methane production mitigation. Twenty lactating Holstein-Friesian dairy cows were divided into two groups, which were offered a total mixed ration fortified with either urea (3.5% DM) or nitrate (a mixture of ammonium and calcium salts, 8.8 % DM). After a 4-week adaptation period, animals were maintained on the same diet for a first monitoring period of 17 days, which was replicated four times at 24-day intervals. MetHb levels measured in the last 4 days of each period were higher in the nitrate group (average range 3.6–4.7%) with respect to urea group (average range 0.4–0.6%); in two out of four periods, maximum MetHb levels approached 20% (Bruning-Fann and Kaneene, 1983).

In another study (Newbold et al., 2014), 36 Holstein steers (288 ± 25 kg bw) were fed corn silage-based total mixed rations with increasing levels of dietary nitrate (0, 0.6, 1.20, 1,80, 2.40 and 3.0% of feed DM) at 4-day intervals for 25 days. Blood MetHb levels were measured on the day after each increase in the dietary nitrate. Nine animals (one from the 1.8% level and four each for 2.4% and 3% levels) were removed from the experiment as they exceeded the MetHb threshold of 20% set by the authors. In contrast to the experiment in sheep of Alaboudi and Jones (1985) described above, despite the adaptation period MetHb was found to increase exponentially in treated steers ranging from about 5% to 25% after the highest nitrate supplementation (3% DM), although none of them displayed clinical signs of nitrate toxicosis. It is suggested that the adaptation period may have increased nitrate reduction to a higher extent than that of nitrite.

Other kinetic studies

A study was performed involving 60 10-week-old Friesian-Dutch bull calves weighing approximately 84 kg. They were divided into a control group and five experimental groups (N = 12 each) fed for 56 days with a milk replacer fortified with 0, 625, 3,233, 8,127 and 16,284 mg potassium nitrate, respectively; the respective added amounts corresponded to a measured concentration of 20, 420, 2,040, 5,520 and 10,060 mg NO_3^-/kg ration. Blood samples were taken at day 0, 1,3,7,14,28 and 56 and nitrate concentration was determined. There was a rapid and dose-related increase in blood nitrate reaching a stead steady from day 7 onwards with values around 3, 15, 60, 150 and 330 ppm, respectively. Treatment withdrawal in the survived animals resulted in rapid fall of the blood nitrate levels which in all groups returned to baseline values within 6 days from treatment cessation (Berende et al., 1979).

Nine male Holstein bullocks (average weight 200 kg) were fasted for 12 h (ad libitum access to water), and randomly allocated to three groups (n = 3/group). For each group fresh pasture (*Pennisetum glaucum*) fertilised with urea (group 1), manure (group 2) or not fertilised (control) was provided once, ad libitum, for 3 h resulting in an average intake of 3.16, 2.98 and 1.67 mg NO_3^-/kg bw, respectively. Blood samples were taken at T = 0, and after 2, 4, 6 and 9 h. Animals from group 1 displayed a time-related significant serum nitrite increase from T4 onwards, rising from around 1 mmol/mL (T0) to more than 1.6 mmol/mL, while no significant differences were noticed between group 2 and control bullocks (Christ et al., 2018).

Twelve Slovak milk-fed spotted calves (unspecified age, initial average weight 43 kg) were allotted to a control group and an experimental group (N = 6 each); the latter was administered with various doses of potassium nitrate for 40 days to develop resistance to nitrate. Urine samples taken from control calves at 1, 10, 20 and 40 days revealed the constant prevalence of nitrate (average around 6.5 mg/L) over nitrite (average around 0.4 mg/L). At the end of the adaptation period, the average body weight was about 63 kg. Three calves from the experimental group received a single dose of 4 g sodium nitrite and the remaining three were dosed once with 30 g potassium nitrate. Urine was sampled every 0.5 h up to 4.5 h. In either group, a prompt urinary excretion of both nitrate and nitrite was recorded, in line with the oxidoreductive reactions involving either compound. In nitrate-administered individuals, NO_3^- urinary concentrations (peak around 2,500 mg/L at 3 h) were higher

than those of NO_2^- (peak around 1,000 mg/L at 1 h). Also, in nitrite-dosed calves, NO_3^- urinary concentrations (peak around 250 mg/L at 2 h) overweighed those of NO_2^- (peak around 70 mg/L at 2 h) (Baranova et al., 2000).

Seven pregnant Holstein-Friesian cows (202–250 day of pregnancy) were equipped with both a tibial arterial and an amniotic fluid catheter and subjected to i.v. infusion (30-min duration) of a 4% sodium nitrite solution at a dosage corresponding to 7, 9.5 or 12 mg NO_2^-/kg bw, with appropriate intervals between each dosing. Blood nitrite concentrations were determined in two cows. There was a rapid decline in maternal nitrite: 135 min after infusion animals dosed with 7 mg NO_2^-/kg bw displayed values below the detection limits (not specified), while the remaining dosages (9.5 or 12 mg NO_2^-/kg bw) resulted in an average nitrite concentration around 10 μmol/L. A clear transfer to fetuses could be demonstrated with fetal plasma values between 10 and 20 μmol/L 105 min after the infusion (Van't Klooster et al., 1990).

The kinetics of nitrite and nitrate after i.v. administration was studied in two groups (N = 6 each) of Polish Merino ewes (1.5 years old, average weight about 44 kg). One group was administered sodium nitrate, while the other received sodium nitrite, in either case at a dose of 400 μmol/kg bw. Blood and urine were sampled at fixed time intervals up to 30 h post-treatment. In nitrite-dosed sheep, there was a complete disappearance of plasma nitrite within 3 h (elimination half-life 0.49 h) along with a parallel increase of nitrate resulting from the oxidation on nitrite and showing a much longer persistence (elimination half-life 4.6 h). A very similar elimination half-life of nitrate (4.5 h) was measured in nitrate-dosed sheep. As regards urine excretion, only about 0.3% of administered nitrite was excreted as such while about 14% was excreted as nitrate. In nitrate-treated sheep, urinary nitrite was not observed while nitrate excretion amounted to about 16% of the administered dose. In case of i.v. administration, the rapid clearance of nitrite from sheep blood plasma points to its almost total conversion to nitrate, while the limited percentage of urinary excretion of nitrate suggest that elimination occurs mainly via other routes (Lewicki et al., 1994).

Pigs

Nitrate salivary recirculation has been studied in 42-day-old piglets (unspecified gender and breed, approximate average weight 12 kg) (Trevisi et al., 2011). In trial 1, six piglets received a single oral dose of 100 g of a commercial diet with 24.5 g KNO_3/kg, containing approximately 130 mg NO_3^-/kg bw. After 2 h, there was a peak in blood and salivary nitrate content showing thereafter a parallel decrease at 6 h along with a progressive increase in nitrite salivary concentration. In trial 2, lasting 14 days, piglets were divided into two groups (N = 3 each). The control group received the basal diet, the treated group was offered the basal diet supplemented with 12.2 g KNO_3/kg, corresponding approximately to 500 mg NO_3^-/kg bw per day. Blood nitrate peaked after 1 week of trial in the treated group (12-fold the control group), then it decreased at the end of the trial, yet maintaining the same ratio with untreated piglets. Conversely, salivary nitrite rose by a factor of seven over control values at 1 week and declined thereafter to 4.5-fold when compared to untreated piglets. It is concluded that salivary recirculation plays an important role in nitrate metabolism in piglets and that the reducing ability of oral microbial populations can be impaired by high amounts of dietary nitrate.

Seventeen pigs of both gender (unspecified age and breed, average weight 12 kg) were maintained under pentobarbital anaesthesia and dosed i.v. (bolus injection) with either sodium nitrate (10 mg/kg bw) or saline. Urine sampling was performed at 60-min interval; after 120 min, animals were euthanised and samples of small intestine, colon, liver and kidney were taken. Urine and tissues were subjected for nitrate and nitrite analysis (HPLC-UV). The urinary nitrate excretion in the treated animals was in the range 434–4,960 μg/h; it could be estimated that approximately 75% of the given nitrate dose was eliminated via urines in the 120-min period (nitrite data not shown). The highest levels of nitrate and nitrite were found in the gut (small intestine and colon intestinal lavages) collectively amounting to approximately 24.8 mg NO_3^-/kg and 0.164 mg NO_2^-/kg, followed by kidney and liver, each displaying concentrations of similar magnitude for either anion (around 24,800 μg NO_3^-/kg and 41 μg NO_2^-/kg). Accounting for the i.v. administration route, the recovery of nitrate in the small intestine together with nitrite is consistent with the enteric excretion of nitrate and its subsequent partial reduction to nitrite by gut bacteria (Eriksson et al., 2018).

Poultry (laying hen, broiler, turkeys, ducks)

No studies concerning the kinetics of nitrate and nitrite in poultry have been found.

Horses

Very little is known about the fate of nitrate and nitrite in horses. The enteric reduction of nitrate to nitrite is believed to occur at a higher rate than in other monogastric species, due to the presence of an active caecal and colonic microbiota (Dicks et al., 2014). In this respect, the enteric nitrate-reducing capacity of horses is said to be intermediate between that of ruminants and the other monogastric species (Bruning-Fann and Kaneene, 1993).

Dogs, cats, fur animals and fish

No relevant data on toxicokinetics have been identified in the literature.

In summary, the main ruminal metabolic pathway involves bacterial NADH- or FADH-nitroreductases mediating a two-step reduction of nitrate, first to nitrite and then (predominantly) to ammonia, which represents an important nitrogen source for bacterial protein synthesis. Nitrate successfully compete with carbon dioxide in accepting the reducing equivalents from NADH or FADH. In so doing, the rumen bacteria limit the biosynthesis of methane, one of the most potent greenhouse gases. The stepwise increase of nitrate in the ruminant diet has been reported to induce adaptive changes of microbial populations; the increase in the conversion of nitrate to nitrite seems to prevail over the conversion of nitrite to ammonia. However, this has limited effects on *in vivo* MetHb formation.

In ruminants, there is a rapid and dose-related absorption of nitrate and nitrite, with a complex interconversion between the two anions followed by a rapid excretion, mainly via urine. The transfer to the fetal blood has been demonstrated in cows.

In pigs, the extent of nitrate reduction to nitrite is much lower than in ruminants. Besides the intestine, it also takes place in the oral cavity due to an extensive salivary recirculation. Little is known about the kinetics of nitrate/nitrite in horses, in which nitrate reduction to nitrite is brought about by an active caecal and colonic microflora and is reportedly intermediate between ruminants and pigs.

No relevant data on the kinetics of nitrate/nitrite in poultry, dogs, cats, fur animals and fish have been identified in the literature.

3.1.1.2. Transfer of nitrite and nitrate from feed to food products of animal origin

Meat and offal

Forty-five Holstein heifers were offered a diet containing sodium nitrate amounting to 0, 440 (approximately corresponding to 2% nitrate in the diet) or 660 mg NO_3^-/kg bw per day, respectively; animals were dosed at three oestrous cycles before breeding or at 40, 150 or 240 days of pregnancy and treatment continued for 30 days after breeding when animals were slaughtered. Muscle samples (*semitendinosus*, *semimembranosus* and *longissimus dorsi*) were collected, frozen and analysed for nitrate content with the method of Greweling et al. (1964) (LOD/LOQ not reported). Nitrate content in muscle showed a treatment-related increase averaging 5, 10 and 21 mg NO_3^-/kg in animals fed 0, 440 and 660 mg NO_3^-/kg bw per day, respectively (Davison et al., 1964). The contents were average values from animals belonging to all the indicated treatment groups at different exposure times except from the 240 days of pregnancy group.

In the study of Berende et al. (1979), sixty 10-week-old Friesian-Dutch pre-ruminant bull calves weighing approximately 84 kg were fed for 56 days with a milk replacer supplemented with potassium nitrate corresponding to a measured concentration of 20 (control), 420, 2,040, 5,520 and 10,060 mg NO_3^-/kg ration. Nitrate and nitrite were determined in liver, kidney and muscle samples with a 'modified Griess method' (detection limits not specified for nitrate, 1 mg/kg for nitrite). A clear treatment-related increase in nitrate content occurred in the order kidney > liver ~ muscle averaging at the two highest nitrate levels 92, 34, 30 and 182, 65 and 65 mg NO_3^-/kg tissue, respectively. By contrast, in tissues from all experimental groups, nitrite content was around the detection limit.

In a large-scale study (Hegarty et al., 2016), a total of 432 composite-meat breed steers (average weight 435 kg at the beginning of the trial) were allotted to four treatment groups receiving diets containing different levels of non-protein nitrogen (NPN), namely 2.5 or 4.5 g N/kg (DM basis) as urea or calcium nitrate (approximately corresponding to 11 or 20 g N- NO_3^-/kg); animals were slaughtered after 102 days on the experimental diets. Neck muscle samples were collected from 4.5 g N/kg treated groups, homogenised and divided into two aliquots (25 g/each), which were, respectively, grilled for 2 min or left uncooked. Samples were then stored frozen for nitrate and nitrite analysis according to

accredited methods. Irrespective of the N source, nitrate was not found in any samples at levels higher than LOD (not specified); nitrite was found only in raw meat samples from both urea- (range 10–13 mg/kg) and nitrate-treated steers (range 9–13 mg/kg).

Milk

In the Davison et al. (1964) study conducted in dairy cows detailed above, milk samples from the cows were collected at weekly intervals for 30 days and analysed by the same method used for muscles. As in the case of muscles, there was a dose-related increase in nitrate content in milk, with average values of 5, 9 and 15 mg NO_3^-/kg in animals fed 0, 440 and 660 mg NO_3^-/kg bw per day dosed cows, respectively (Davison et al., 1964).

The most recent studies in dairy cows were designed to verify the use of nitrate supplementation to mitigate methane emissions. Holstein dairy cows were fed a ration with or without 24 g calcium ammonium nitrate/kg DM (18 g NO_3^-/kg DM) corresponding to approximately 127 mg NO_3^-/kg bw for 119 days. The cows' milk (collected once a week in weeks 5, 9, 13, and 17) had no measurable nitrate and nitrite levels (values from untreated cows not reported). The milk samples were analysed by an official method ('spectrometry') with an LOQ of 5 mg/kg for nitrate and 0.7 mg/kg for nitrite (Guyader et al., 2016).

Lactating Jersey dairy cows were offered a diet with or without calcium ammonium nitrate (± live yeast culture (LYC)). Cows were adapted to the nitrate diet by feeding 5 g NO_3^-/kg DM for 3 d, followed by 10 g NO_3^-/kg DM for 4 days and started being fed 15 g NO_3^-/kg DM at the beginning of the second week. At the end of the 4-week experimental period NO_3^- and NO_2^- were determined in milk samples by an EPA method (not described). There was an increase in nitrate milk content from cows fed NO_3^- (0.357 mg NO_3^-/L in control cows vs. 0.537 mg NO_3^-/L), the nitrite content being below the detection limits (LOQ not reported). LYC supplementation had no effects on NO_3^- and NO_2^- contents (Meller et al., 2019).

Four lactating Danish Holstein dairy cows were allotted to four calcium ammonium nitrate addition levels, namely 0, 5.3, 13.6 and 21.1 g of NO_3^-/kg of dry matter (DM). After a 6-days period of gradual introduction of nitrate, cows were offered the experimental diets for 16 days before sampling. Nitrate and nitrite concentrations in milk were determined according a spectrophotometric method (LOQ not specified, LOD of 30 μg mentioned for nitrite only). There was a dose-related linear rise in nitrate concentrations in milk from 0.13 to 1.56 mg/L, while nitrite concentrations were below the LOD (< 30 μg/L) (Olijhoek et al., 2016).

Eggs

Animals (25-week-old SexSal laying hens (average production 9 eggs/10 days) were allotted to a control group (N = 2) and six experimental groups (N = 4 each) receiving commercial diets supplemented with 100, 1,000 or 5,000 mg N-nitrate or nitrite (potassium salts)/kg for 10 days, respectively; the unsupplemented (control) diet was offered for further 5 days. Eggs were regularly collected for the entire duration of test and analysed for nitrate/nitrite content by a GLC-ECD method (Tanaka et al., 1983). The same method was used to analyse 50 eggs collected from the market, which showed a nitrate content (range 0.052–0.076 mg/kg) about twice that of nitrite (0.026–0.034 mg/kg). Nitrate and nitrite contents in eggs from hens receiving the lowest supplementation (100 mg potassium nitrate/nitrite) were similar to those from control animals or from commercially collected eggs. The dietary exposure to higher potassium nitrate dosages (1,000 or 5,000 mg/kg) resulted in the accumulation of both nitrate and nitrite, reaching a steady state with a peak level around 1.7 mg/kg for nitrate and less than 0.1 mg/kg for nitrite for both anions after 2–3 days. The levels rapidly declined upon treatment cessation to return at baseline levels in 5 days. When potassium nitrate was replaced by potassium nitrite, the steady state for both anions was reached 3–4 days after the beginning of the treatment with a peak level around 1.5 mg/kg for nitrate and around 0.9 mg/kg for nitrite; a rapid decline upon treatment withdrawal was also observed for both anions again reaching pre-treatment values in 5 days. Although little is known about nitrate/nitrite metabolic fate in poultry, the results of this study would indicate that in the laying hens, the extent of the oxidation of nitrite to nitrate outweighs the extent of the reduction of nitrate to nitrite. It remains to be established whether these reactions occur (also) in eggs.

In summary, there is scant information concerning the transfer of nitrate or nitrite from feed to animal food products. A dose-related accumulation of nitrate and nitrite in animal products from experimentally treated animals has been demonstrated. However, based on a very limited database, a calculated transfer factor of less than 0.001 for nitrate or nitrite in meat, offals, milk and eggs can be estimated.

3.1.2. Mode of action

The nitrate itself is less toxic than the nitrite, which causes the formation of MetHb, a molecule with very limited oxygen carrying capacity. Acute toxicity is seen more often than chronic effects and mainly reflects tissue oxygen deprivation. Clinical symptoms include anoxia, tachycardia, dyspnoea, muscle tremors, reduction in blood pressure, weakness, vomiting, unstable gait, cyanosis (exhibited by brown-coloured arterial blood), polyuria, lethargy and death (Bruning-Fann and Kaneene, 1993; EFSA, 2009; Wallig et al., 2017). The sudden fall in blood pressure associated with acute poisoning might be caused by nitric oxide radical ($NO^{\cdot}$) which is formed from elevated concentrations of nitrite in the gastrointestinal tract as well as in the circulation and in tissues. Petechial haemorrhages in the epicardium, endocardium, peritoneum, mucous membranes of the respiratory tract and rumen and the serosal surface of the digestive tract are frequently found on necropsy (Bruning-Fann and Kaneene, 1993). Symptoms of subchronic and chronic toxicity include reduced feed intake, decreased milk production (dairy animals), rough hair, loss of weight or no weight gain, lowered vitamin A and E levels, impaired fertility and abortion.

3.1.2.1. Methaemoglobin formation

Increase of methaemoglobin levels is considered the principal mediator leading to toxicity of nitrate and nitrite in animals. Nitrite is considered the toxic chemical species and toxicity of nitrate is mainly caused by bacterial conversion of nitrate to nitrite in the gastrointestinal tract. The rate of conversion of nitrate to nitrite therefore dictates the toxicity of an animal to nitrate. This rate varies among species and within species. In ruminants, the microbiome of the rumen shows adaptive changes over time in response to increased nitrate intake reportedly enhancing the tolerance of the animal to nitrite. Actually, these changes seem to reflect an increase in the nitrite reducing capacity with little effects on the actual amount of MetHb formed (Section 3.1.1.1 on *Nitrate and nitrite adaptation*). The mechanisms of the nitrite-induced MetHb formation are complex and involve the generation of reactive intermediates, such as nitrogen dioxide radical ($NO_2^{\bullet}$) and superoxide anion ($O_2^{-}\bullet$) (Tomoda et al., 1981; Titov and Petrenko, 2005). Consequently, the antioxidant defence systems of red blood cells (e.g. GSH, ascorbic acid, catalase, SOD, GSH-Px) and an efficient reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system play a key role in limiting MetHb generation (Titov and Petrenko, 2005; Franco et al., 2019). In this respect, it should be noted that ascorbic acid (1–9 mM) was able to reduce the *in vitro* $NaNO_3$ 3 mM-mediated MetHb formation in rat and human erythrocytes, but not in ovine erythrocytes (Calabrese et al., 1983) Tolerance to nitrate/nitrite toxicity is also dependent upon the activity of erythrocyte MetHb reductases which can convert MetHb back to haemoglobin. In both mammalian and fish species, this is primarily accomplished by the flavoprotein cytochrome b5 reductase, catalysing the electron transfer from reduced nicotinamide adenine dinucleotide (NADH) first to cytochrome b5 and then to the ferric haem of MetHb resulting in the regeneration of fully functional Hb (Jaffé, 1981; Jensen and Nielsen, 2018). There is evidence indicating an increase in this NADH-dependent MetHb reductase in red blood cells from steers chronically exposed to nitrate (Godwin et al., 2015).

Another erythrocyte enzyme, referred to as NADPH-dependent MetHb reductase or diaphorase, is also active in reducing MetHb. It is expressed also in fish (Saleh and McConkey, 2012) and comparable activities have been shown in human, horse, cat and dog erythrocytes (Harvey and Kaneko, 1975). However, it requires an intermediate electron acceptor (e.g. methylene blue or various flavins) for being effective; therefore, NADPH-dependent MetHb reductase is considered of minor importance in tackling MetHb formation (Harvey, 2008), as recently confirmed in nitrate-exposed cattle (Godwin et al., 2015).

No major differences in the basal activity of erythrocyte NADH-dependent MetHb reductase were noticed between monogastric (horses and pigs) and ruminant species (Lo and Agar, 1986). Considerable interspecies variation in the *in vitro* ability to reduce sodium nitrite-induced MetHb formation has been reported, with ruminants and pigs as the most and the least active, respectively (Smith and Beutler, 1966; Cockburn et al., 2013). The relative inefficiency displayed by pig erythrocytes is likely due to the relatively low ability of their membranes to take up glucose, the major energy source for MetHb reduction (Kwong et al., 1986).

In the previously cited study by Lo and Agar (1986), higher values of NADH-dependent MetHb reductase were consistently found in newborn as compared to adult individuals of all tested species but rabbits and humans.

In summary, methaemoglobinaemia is the major adverse effect resulting from acute nitrate/nitrite animal exposure, nitrite being the causative agent. Interspecies differences in the rate of MetHb formation are mainly related to the extent and the rate of nitrate reduction to nitrite, which is higher in ruminants, intermediate in horses and lower in the other monogastric species. The erythrocyte NADH-dependent cytochrome b5 reductase (NADH-MetHb reductase) is the most active enzyme system in the inactivation of MetHb, while NADPH-dependent MetHb reductase is of minor importance. Pig erythrocytes are less able to convert nitrite-mediated MetHb formation because of their poor ability to take up glucose, which is the major energy source for MetHb conversion to Hb. MetHb formation is also affected by age (fetuses and neonates are more sensitive) and the antioxidant status, while in ruminants, progressive adaptation to increased exposure of nitrate plays a minor role.

3.1.2.2. Changes in microbial populations

In ruminants, a gradual increase in dietary nitrate intake causes an adaptive selection of microbes favouring those with high ability in converting nitrate to nitrite and nitrite to ammonia (see 3.1.1.1) (Yang et al., 2016). In addition, *in vitro* studies (reviewed by Latham et al., 2016) indicate that the direct addition of nitrate to cattle ruminal fluid brings about selective changes in the microbial populations. In particular, the resulting increase in nitrite may lower microbial populations of the cellulolytic bacteria, which are involved in fibre degradation and provide volatile fatty acids and protein for the host animal. This effect has been associated with an increase in dry matter retention time in rumen and a consequent decrease in dry matter intake (Allen, 2000), which has been detected in nitrate exposed cows (Meller et al., 2019). On the other hand, dry matter intake was found unaltered in other *in vivo* studies on nitrate-adapted bovines, suggesting a limited effect of nitrate on cellulolysis (reviewed by Lee and Beauchemin, 2014).

In summary, several in vitro investigations have pointed to a negative effect of nitrate and nitrite on cellulolytic microbes. However, the practical impact of these effects under *in vivo* conditions remains to be established.

3.1.2.3. Thyroid effects

The repeated exposure to dietary nitrate has the potential to affect thyroid function by acting as a competitive inhibitor of the sodium-iodide symporter (NIS), leading to a decrease in both iodide uptake and its availability for thyroid hormone synthesis (Pearce and Braverman, 2009). The rat is considered one of the most sensitive species to nitrate-mediated thyroid effects (Zaki et al., 2004). Regarding ruminants, few relatively old studies (reviewed by Bruning-Fann and Kaneene, 1993) point to negative effects of nitrate on thyroid function in cattle and sheep, with an indication of a decrease in plasma protein bound iodine, thyroid ^{131}I uptake and T4 levels. More recently, a significant decrease in the circulating levels of T4 was detected in bulls exposed to increasing dosages of nitrate (potassium nitrate) for 30 days starting from 100 g/head and increased at weekly intervals up to 250 g/head (Zraly et al., 1997). In other studies, adverse effects on thyroid function tended to disappear toward the end of the experimental period, possibly reflecting adaptation to nitrate (Arora et al., 1968). This interpretation is consistent with the lack of effects on thyroid weight documented in dairy cows exposed to 440 or 660 mg nitrate ion per kg bw before or at 40 days or 150 of pregnancy and slaughtered 30 days post-calving (Jainudeen et al., 1965). No significant effects on circulating thyroid hormones were also reported in Angora goats offered a diet supplemented with 1,500 mg/kg nitrate for 180 days (Avci et al., 2018). These results are in contrast with a recent report on a dairy cattle herd with a history of reproductive problems (abortion, premature and stillborn calves) due to nitrate levels in water and feed up to 390 mg/kg. Colloidal goitre was consistently detected in stillborn calves, while the free T3 and T4 concentrations and the total T4 concentrations were significantly reduced in all pregnant cows (Sezer et al., 2011).

Concerning monogastric species, goitrogenic effects upon repeated dietary exposure were observed in chickens (4 g NO_2^-/kg diet) but not in turkeys (675 mg NO_3^-/kg in the drinking water) or in dogs (up to 1,000 mg/kg NO_3^- in the drinking water for one year) (reviewed by Bruning-Fann and Kaneene, 1993). The dietary supply of 30 g KNO_3/kg for 5 weeks to piglets (breed, gender and age not reported) lead to a marked decrease in T4, T3 and rT3 serum concentrations. The inclusion of a high dietary iodine supplementation (1 mg/head) for a further week was able to reverse the altered values of thyroid parameters, confirming the iodine-responsive nature of nitrate effects on thyroid (Jahreis et al., 1986).

Contrasting results have been reported in fish. Waterborne exposure to moderate water NO_3^- concentrations (1.5 mg/L) – for 64 days resulted in significantly lower iodine-125 (^{125}I) uptake by a

number of tissues including thyroid in the perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*) and Crucian carp (*Cyprinus carassius*); the resulting effects on circulating thyroid hormones, however, were not reported (Lahti et al., 1985). Whitespotted bamboo sharks (*Chiloscyllium plagiosum*) raised in a 70 mg/L NO3-N seawater for 29 days did not experience a significant reduction in plasma-free thyroxine (FT4), but did show signs of mild to moderate hyperplasia and hypertrophy of the thyroid gland (Morris et al., 2011).

In summary, under conditions of repeated dietary exposure, nitrate may act as a competitive inhibitor of NIS thereby decreasing iodine uptake and thyroid hormone synthesis. In ruminants, an overt impairment of thyroid function has been reported under field conditions, while the attempts to experimentally reproduce the syndrome have been in some cases unsuccessful, likely due to a progressive adaptation to nitrate. Thyroid function depression has been experimentally reproduced in chickens and piglets but not in turkeys and dogs.

3.1.2.4. Nitrate/nitrite-induced oxidative stress

As previously mentioned, under the acidic conditions of the stomach, nitrite is rapidly converted to nitrous acid, which spontaneously decomposes to various nitrogen oxides, including nitric oxide ($NO^{\bullet}$) (Bryan and Lancaster et al., 2017; Hancock and Neill, 2019). In turn, $NO^{\bullet}$ may react with superoxide anion $O_2^{-\cdot}$, arising from many physiological processes. The nitrite-mediated MetHb formation results in formation of the highly reactive peroxynitrite free radical ($ONOO^-$) (Tomoda et al., 1981). This potent pro-oxidant is implicated in a variety of cytotoxic effects including lipid peroxidation, oxidation of protein and non-protein –SH groups, protein nitrosylation and several others leading to the impairment of the antioxidant defence, of vital cell functions (e.g. mitochondrial respiration) and ultimately to cell death (for a review, see Ahmad et al., 2009). Nitrate/nitrite-mediated oxidative stress has been reported in Friesian-Holstein cows from a herd with a history of nitrate exposure for more than 6 months via contaminated feed and water. Animals showed increases in plasma thiobarbituric acid reactive substances (TBARS) along with a decrease in GSH content and in SOD, GSH-Px and catalase plasma activities, with blood MetHb content being only slightly increased with respect to an appropriate control (Al-Qudah, 2010). Blood changes indicative of oxidative stress (increase in TBARS, decrease in SOD and catalase activities) along with a rise in blood nitrite have been documented in bullocks fed on pasture high in nitrate content (Christ et al., 2018).

In summary, the metabolic fate of nitrite entails the direct or indirect generation of a number of reactive species, including $NO^{\bullet}$ and the $ONOO^-$, which are responsible for cell oxidative damage (lipid peroxidation, oxidation of protein- and non-protein sulfhydryls) and cytotoxicity. Plasma changes consistent with an increased oxidative status have been found in cattle with high dietary exposure to nitrate.

3.1.2.5. Effects on Vitamin A and Vitamin E content

Research published in the 1960s and 1970s and reviewed by Bruning-Fann and Kaneene (1993) provides evidence that feeding cattle, sheep, pigs or chickens with diets high in nitrate or nitrite content may result in decrease in liver and plasma vitamin A content. While contrasting results have been reported for ruminants and pigs, this was seemingly not the case for poultry (chickens and turkeys), in which the experimental exposure to various concentrations of nitrite (starting from 200 mg/kg) consistently lowered the body stores of vitamin A and/or β-carotene. The proposed mechanisms include mainly a direct destruction of the vitamin A or of β-carotene, with nitrite and nitric oxides being more active than nitrate in this respect. A decrease in the absorption of vitamin A and β-carotene and the impairment of the conversion of β-carotene into vitamin A have been also proposed. Finally, the observed nitrate-dependent depression of thyroid hormone synthesis might be also implicated, insofar as thyroid hormones are thought to affect vitamin A body stores in the body. More recently, during field outbreaks of chronic nitrate toxicosis in cattle characterised by abortion, low fertility and thyroid enlargement, affected cows had a marked fall in both serum vitamin A and β-carotene levels with respect to healthy individuals (Sezer et al., 2011).

Vitamin E is thought to afford protection against the adverse effects of nitrite by reducing the formation of $ONOO^-$ and/or limiting the $ONOO^-$ -mediated peroxidative damage (Chow and Hong, 2002). Although nitrate/nitrite exposure has been historically associated with depletion of vitamin E stores (Ridder and Oehme, 1974), limited research has been done to address this issue. In pigs, an inverse relationship was found between the levels of administered nitrite and the vitamin E status (London et al., 1967). A remarkable decrease in vitamin E serum levels has been reported in one study

in bulls exposed to increase nitrate dosage levels for 30 days (Zraly et al., 1997, see above); however, no conclusive evidence has been reported for ruminants.

In summary, there are reports indicating that the prolonged exposure to nitrate and nitrite is associated with depletion of vitamin A stores with mechanisms still to be defined. Nonetheless, no conclusive evidence has been provided for ruminants and pigs while limited evidence is available for poultry. Evidence has been provided supporting the protective role of vitamin E against the nitrate/nitrite-mediated oxidative damage in laboratory species. No clear conclusions for farm and companion animals may be drawn, given the limited research performed in such species.

3.1.2.6. Reproductive effects

As detailed in the previous EFSA Opinion (EFSA, 2009), adverse effects on reproduction may occur following chronic exposure to nitrate (or nitrite) and are mostly observed in cattle under field conditions. Reduced fertility, late abortion and also stillborn and parturition of premature individuals have been described in cows, although this picture could not be fully reproduced in experimentally treated individuals (Davison et al., 1964; Winter and Hokanson, 1964). The negative effects on reproduction are likely to be the result of nitrate/nitrite exposure in combination with a number of associated effects. In short, they would include depression of thyroid function, inhibition of steroidogenesis (particularly progesterone), decrease in the antioxidant status (β-carotene, vitamin A, vitamin E, ascorbic acid) as well as decrease in fetal oxygen supply due to a progressive rise in MetHb content (EFSA, 2009). Fetal Hb is more sensitive to nitrite compared to the maternal one because fetal haemoglobin ($\alpha 2\gamma 2$) forms MetHb more readily than the adult form ($\alpha 2ß2$) (Malestein et al., 1980; Greer and Shannon, 2005).

It has more recently been observed that cows with a history of nitrate-related abortion exhibit a marked depression in blood total and free T4 as well as in blood β-carotene and vitamin A levels; this provides further support to the involvement of thyroid dysfunction and compromised (pro)-vitamin A status in the pathogenesis of nitrate-mediated miscarriage (Sezer et al., 2011). In addition, outbreaks of reproductive losses, abortion and delivery of neonates with enlarged thyroids have been documented in mares and goats chronically exposed to high dietary nitrate (Swerczek and Dorton, 2019).

It is generally accepted that the repeated exposure to nitrate/nitrite may induce abortion and negatively affect the reproductive performance of several species, despite that some experimental studies failed in reproducing the clinicopathological picture described in field outbreaks. The main mechanisms underlying these effects include methaemoglobinaemia and decreased fetal oxygen supply, depression of thyroid function and decrease in the antioxidant status.

3.1.2.7. Effects on blood pressure

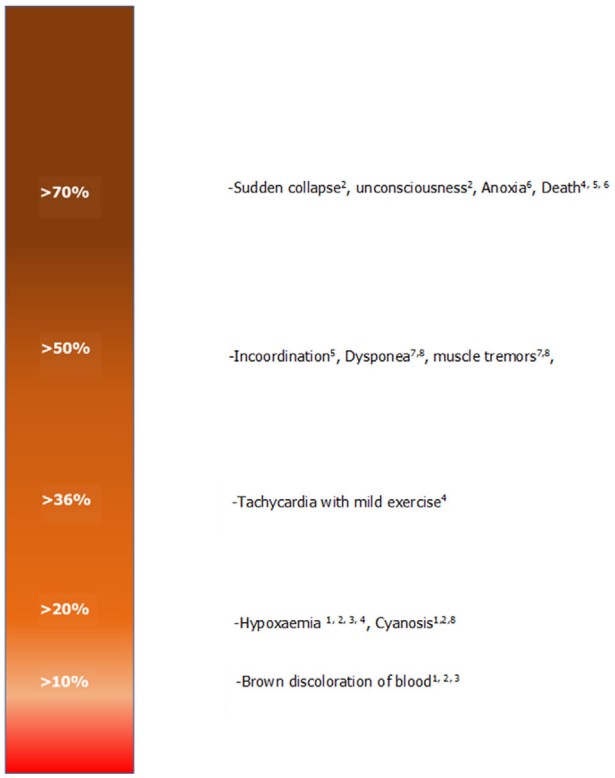
A common finding of the acute exposure to nitrate/nitrite is the fall in blood pressure. This may contribute to the development of MetHb-induced tissue anoxia due to a peripheral circulatory failure resulting from vasodilation. Until few decades ago, these effects were believed to be entirely due to nitrite itself. It is now clear that $NO^{\bullet}$ plays the major role in triggering blood pressure fall (Lundberg and Weitzberg, 2010). Nitric oxide can arise both from nitrite reduction by a variety of not yet clearly understood pathways and from endogenous metabolism. As the nitrite-mediated $NO^{\bullet}$ generation is known to augment during tissue hypoxia (Lundberg and Weitzberg, 2010), the resulting hypotensive effects would likely be more severe if the blood MetHb content is high.

3.1.3. Adverse effects in farmed and companion animals

3.1.3.1. Adverse effects in ruminants

The generation of MetHb from the reaction between nitrite and oxyhaemoglobin is well established and is considered the main mediator of most sensitive adverse effects following exposure to nitrate and nitrite in ruminants (EFSA, 2009). The consequential depression of aerobic metabolism can be quantitatively associated with many clinical signs, such as cyanosis, hypoxaemia, tachycardia, dyspnoea, incoordination, muscle tremors and death (Figure 1). Although direct evidence is lacking, MetHb formation could be plausibly linked to cases of abortion and still birth (Ashbury and Rhode, 1964; Burrows et al., 1987; Issi et al., 2008; Vermunt et al., 2010; Benu et al., 2016, 2018; Benu, 2017). The association between production of MetHb in the blood and reported clinical signs is illustrated in Figure 1. However, the Panel notes that few limited field studies from Turkey (Ozmen et al., 2005; Sezer et al., 2011), with incomplete reporting of adverse effects in cows for reproduction,

describe reproductive effects at low MetHb levels (systematic measurement missing), suggesting that a variety of factors (vitamin A and vitamin E antioxidant status, thyroid function, oxidative stress) might contribute to the outcome for this animal category.



[1]Benu, 2017; [2]Benu et al., 2016; [3]Benu et al., 2018; [4]Vermunt et al., 2010; [5]Burrows et al., 1987; [6]Ashbury and Rhode, 1964; [7]Issi et al., 2008; [8]Al-Qudah et al., 2009.

Figure 1: Association between metHb% in blood and reported clinical sings of nitrate toxicity in cattle

It is difficult to determine safe intake levels for nitrate and nitrite because of the variety of factors affecting their potential toxicities (Leng, 2008). Several factors contribute to the susceptibility of the individual ruminant species and animals to nitrate toxicity. This includes the nitrate/nitrite levels consumed by the animals that can be very different depending on the dietary sources of feed (grazing, feeding on hay and silages and other sources) (Geurink et al., 1979). Other factors that contribute are the dietary nitrate/nitrite consumption rate and the rumen nitrate and nitrite reduction activity and capacity, which influence the conversion rates of nitrate to nitrite. Notably, as discussed in Section 3.1.1.1 above, ruminal adaptation to nitrate and nitrite through gradually increasing their daily doses (a process referred to as 'training') may have the potential to increase tolerance in ruminants (Leng, 2008) and in particular in sheep (Alaboudi and Jones, 1985).

Consideration of setting separate tolerable nitrate doses for adapted and non-adapted ruminants

Because of the potential beneficial consequence of feeding ruminants with elevated levels of nitrate on methane reduction, the CONTAM Panel considered the possibility that different tolerable levels for nitrate could be set for adapted and non-adapted bovines. Therefore, a brief review was made of the strategies employed to avoid poisoning from feed containing elevated levels of nitrate. Identified studies on feeding strategies to maintain asymptomatic MetHb levels are summarised in Table A.1 in Appendix A. Typically, the strategies come from studies focused on the reduction of methane emissions in cattle and include 1) fractionating the total dose of nitrate into two or more equal doses throughout a day, 2) gradually increasing the amount of nitrate in the diet, 3) providing nitrate in an encapsulated form and 4) feeding nitrate evenly distributed throughout a feed ration. According to van Zijderveld et al. (2011), MetHb levels in cattle remain below 5% at a dietary dose of nitrate of 680 mg/kg bw per day, included in a total mixed ration. However, cattle consuming feed with nitrate at these levels tend to have decreased feed intake from 4% to 16% (Hulshof et al., 2012; Newbold et al., 2014; Velazco

et al., 2014) (Appendix A, Table A.1). Benu, 2017, dosed 3-year-old *Bos indicus* steers (average bw = 400.7 kg) with nitrate at a daily dose of 125 mg/kg bw (50 g nitrate/animal per day) delivered into the rumen via a fistula for 70 days and measured the MetHb content of the blood at time intervals after the dosing. The blood MetHb concentration peaked at about 18% after each dose and was remarkably constant during the 70-day trial. This indicates that adaptation to dietary nitrate did not occur under the conditions of the study by Benu (2017), and therefore, tolerance to dietary nitrate does not reliably increase following long-term exposure.

A pooled analysis addressed the quantitative relationship between a single dose of nitrate and MetHb formation by conducting an analysis of data from nine studies with a total of 25 treatments, including two studies in beef, two in dairy cows and also five studies in sheep (Lee and Beauchemin, 2014). This study found a highly significant linear increase in the percentage of MetHb in blood as a function of nitrate dose: MetHb (%) = 41.3 × nitrate (g NO_3^-/kg bw per day) + 1.2 (R^2 = 0.76, $p < 0.001$). However, the responses of the animals included in this regression analysis were all 40% MetHb or lower and corresponded to the linear part of the sigmoidal dose-response curve proposed by Crawford (1965). It was noted that two of the data points for control animals in the analysis by Lee and Beauchemin (2014) showed negative percentages of MetHb, which is difficult to interpret. Lee and Beauchemin (2014) analysed a much more limited data set composed of two studies in dairy cows and one in sheep. They found that in ruminants adapted to a high nitrate diet, the dose-response between a single dose of nitrate and the percentage of MetHb in the blood was much shallower, than that obtained in non-adapted animals. At doses between 650 and 700 mg/kg bw, MetHb increased to less than 5%, compared to about 40% at a similar dose in non-adapted cattle and sheep. According to the combined analysis done on non-adapted cattle and sheep, the doses causing an average increase in blood MetHb by 5% and 10% would be 92 and 210 mg NO_3^-/kg bw.

The CONTAM Panel considered that although there are many examples in the literature indicating successful adaptation to nitrate in feed, the outcome is variable and, therefore, there are insufficient grounds to set a different maximum tolerable intake of nitrate for ruminants, which have undergone long-term exposure to elevated levels of nitrate.

Consideration of critical effect

The CONTAM Panel reviewed the new literature available and confirmed that for most categories of ruminants, methaemoglobinaemia is the critical effect of nitrate and nitrite from which most other clinical signs are derived.

Bovines

Adult bovine

As discussed above, an increase in the percentage of MetHb in blood was selected as the critical effect in cattle. Although the opinions of different authors deviate, new literature reviewed by the CONTAM Panel suggests that there is limited evidence for clinical signs occurring in most ruminant species and categories when MetHb levels remain below 10% (Benu et al., 2016; Benu, 2017; Benu et al., 2018). Therefore, this value was used to define the benchmark response for cattle.

In order to derive a reference point for nitrate in cattle that is protective for all feeding regimes, the CONTAM Panel considered oral dose-response studies involving direct feeding of nitrate, once a day, to non-acclimated cattle, with post-prandial MetHb measurements. Three particularly useful publications were identified, authored by Crawford et al. (1966) and Benu et al. (2016, 2018).

Crawford et al. (1966), combining data from several studies, found a sigmoidal relationship between nitrate dose and percentage MetHb in non-acclimated cattle. There was a clear time dependency of the effect with a peak in methaemoglobinaemia occurring between 2- and 4-h post dose. Regression analysis suggested that a 10% MetHb percentage coincided with a nitrate dose of approximately 80 mg/kg bw. A subset of the data from Crawford et al. (1966) were re-analysed by Leng (2008) showing that a small increase in intake of nitrate can result in an exponential increase in blood MetHb. The CONTAM Panel noted that the control animals were reported to have an average high MetHb content of 5%, that all doses applied resulted in > 10% MetHb, and that the response was extremely variable in doses at and above 250 mg NO_3^-/kg bw.

In a study by Benu et al. (2016), 12 *Bos indicus* steers (~ 320 kg bw) were fed daily nitrate supplement doses of 0, 30, 40 or 50 g of nitrate per day (approximate doses: 0, 94, 120, 160 mg NO_3^-/kg bw per day) with a feeding frequency either once or twice a day for a week. In this study, the mean MetHb content of the control animals was about 2% of total Hb. In animals fed 94 mg NO_3^-/kg

bw per day, daily postprandial peak MetHb values were relatively stable with adjusted mean daily peak values of between 7% and 11% MetHb from Day 1 to Day 7. The dose rates of 120 and 160 mg NO_3^-/kg bw per day resulted in adjusted mean daily peak MetHb concentrations that increased over time from 21% and 28% on Day 1 to 53% to 59% on Day 7, respectively. It can be estimated that a 10% increase in MetHb concentration would be expected at intakes higher that 94 mg NO_3^-/kg bw per day (see BMD calculations below).

In a related study (Benu et al., 2018), 2-year-old *Bos indicus* steers (n = 12, average bodyweight = 397 kg) were dosed once per day through an indwelling rumen catheter with 0, 76 or 130 mg NO_3^-/kg bw per day for 7 days. On Day 7, the animals were exercised through walking 3 km before sampling. MetHb content of the blood showed a dose-dependent increase with nitrate doses, and was 0.3% (control), 8.6% (76 mg/kg bw per day) and 32.9% (130 mg/kg bw per day). There were also dose-dependent reductions in percentage of oxyhaemoglobin and the partial pressure of the oxygen of the blood, and an increase in carboxyhaemoglobin, but these changes were only significant at the highest dose. Likewise, there was a dose-dependent increase in the heart rate after the exercise, and this was statistically significant in the animals given 130 mg NO_3^-/kg bw per day.

Lactating cows

Feeding of Holstein-Friesian dairy cows (average bw ~ 590 kg calculated from metabolic bw 120 kg) for four 24-day periods with a complete feed (average ~ DMI 19 kg per day) containing 21 g NO_3^-/kg (dry matter) corresponding approximately to 680 mg/kg bw per day resulted in 4–5% MetHb. Although the blood MetHb content of treated animals was modest, it showed no sign of recovery up to 91 days of nitrate feeding with total Hb levels being increased above the urea control throughout the trial (van Zijderveld et al., 2011). The cows receiving elevated nitrate had increased metabolisable energy, no effect on milk yield and a decrease of milk protein content by 5%.

Four lactating Danish Holstein dairy cows (average bw ~ 595 kg) were fitted with rumen, duodenal and ileal cannulas and assigned to groups with four calcium ammonium nitrate addition levels, 0, 5.3, 13.6 and 21.1 g of NO_3^-/kg of dry matter (DM), resulting in approximate doses of 0, 150, 425 and 650 mg NO_3^-/kg bw per day (DMI 17, 17.3, 18.7 and 18.5, respectively). The diets were isonitrogenous, by replacing urea for nitrate. Animals were gradually introduced to the experimental diets over a 6-day period and were then allowed to acclimate to the nitrate doses for another 10 before the sampling period which lasted from day 17 to day 21. The nitrate concentration of milk increased linearly with dose from 0.13 to 1.56 mg/L with nitrite levels below the LOD (< 30 μg/L). Blood MetHb content increased dose-dependently from 1.3% in the control to 1.65% in the high-dose group, which was the only condition statistically different from the control. There were no effects on milk yield, milk composition including protein, DMI and digestibility of DM, organic matter, crude protein and neutral detergent fibre in rumen, small intestine, hindgut and total tract (Olijhoek et al., 2016).

Multiparous Jersey cows (average bw 408 kg) were acclimated for 3 weeks to increased dietary nitrate levels (n = 20), followed by 8 weeks on 0, 11 and 23 g of NO_3^-/kg (DM) corresponding approximately to 0, 150 or 310 mg NO_3^-/kg bw per day, given in concentrate feed (DMI of concentrate 5.4 kg/day). Cows exposed to 314 mg NO_3^-/kg bw per day showed 12% lower milk yield and 13% lower fat milk than the control with no effects observed in the 150 mg/kg bw per day group for milk protein (van Wyngaard et al., 2018). Reduced protein content of milk by 9% was also observed in Jersey cows (default bw average 454 kg – see NRC, 2001) fed diet containing 15 g NO_3^-/kg feed DM (corresponding approximately to 570 mg/kg bw per day with a DMI of 17.2 kg DMI), compared with controls (Meller et al., 2019). In this study, there was also a 5.5% reduction in feed intake, but only a small effect on blood MetHb content (1.6% in nitrate exposed cows compared with 0.5% in controls).

Overall, an effect on milk quality parameters (reduced protein and fat content) can occur when lactating cows are fed high doses of nitrate.

Young bovines

Baranova et al. (1999) noted no clinical signs in bull calves (~ 75 kg bw) exposed first for 6 weeks to 2 or 5 g potassium nitrate daily (23 or 58 mg NO_3^-/kg bw per day; average bw 52.5 kg) and then to 5 or 10 g daily (36 or 72 mg NO_3^-/kg bw per day; average bw 85 kg), for additional 20 days, via oral administration of an aqueous solution. The average MetHb content reached a maximum average of 3.96% during the study (Baranova et al., 1999). Exposure of 10-week-old Friesian-Dutch calves (~ 84 kg bw) to nitrate doses between 0 and 10 g/ kg milk replacer (0 and 230 mg NO_3^-/kg bw per

day in milk replacer; average bw 134 kg, average ration 3.04 kg/day) was reported to have no adverse effects (Berende, 1977).

Seven 3- to 6-month-old calves of black–white and Slovak spotted breeds (average 97 kg bw), which were on solid feed, were administered a single dose of 310 mg NO_3^-/kg bw directly into the rumen (Nagy et al., 2013). Blood MetHb levels peaked 4–6 h after the dosing with an average of 27%. The highest MetHb levels were observed in the three youngest calves (67–90%). Moderate cyanosis was evident at 20% MetHb. Reduced feeding and depressed activity were observed in calves with MetHb above 40% and more severe effects were apparent in individuals with MetHb above 60%, including ataxia and muscle tremors. MetHb content of the blood was correlated positively with pulse rate, breathing rate and to blood pH and negatively correlated with body temperature and oxygen tension, carbon dioxide tension, oxygen saturation and bicarbonate concentration of the blood.

The potential effects of nitrate in water for drinking were investigated in 3-day-old Slovak spotted bullocks (Jacková et al., 1992). The animals were 3 days old and weighed 40 kg at the start of the experiment. All animals were fed colostrum for the first 5 days, followed by milk replacer alone up to an age of 1 month, and then milk replacer supplemented with formic acid as a preservative. One group of six calves were kept as controls, and the other group of six calves given a daily oral dose of nitrate, starting with 613 mg daily (15 mg NO_3^-/kg bw; 40 kg bw calves) and then incrementally increased weekly to a final dose of approximately 6.130 mg daily (~ 100 mg NO_3^-/kg bw per day (final weight estimated to 60 kg from Baranova et al., 2000). Blood samples were collected before and 1, 2, 3 and 4 h after the nitrate dose. The MetHb content of the control group was on average 1.6% throughout the experiment. Blood MetHb content peaked 2–3 h after each dosing and increased to 2.2% (from 1.6% before administration) at the first sampling point 5 days into the experiment, when the dose could be determined to 15 mg NO_3^-/kg bw per day. The MetHb percentage increased dose-dependently to a maximum of 3.6% at the highest dose (100 mg NO_3^-/kg bw per day). In this study, urinary NO_3^- and NO_2^- was also measured. Interestingly, the molar ratio between urinary NO_3^- and NO_2^- declined from the 1 h to the 4 h sampling points after administration. The urinary NO_3^-/NO_2^- ratio was also sixfold higher after administration of the final dose compared with the initial.

A study was carried out in 10-week-old Holstein male calves investigating the effect of nitrite on the performance and vitamin A and carotene metabolism (Cunningham et al., 1968). Nitrite dosing at approximately 4,400 mg KNO_2 /100 kg bw (24 mg NO_2^-/kg bw per day) with or without either vitamin A (44 or 88 I.U. vit A/kg bw per day) or carotene (44 I.U. (88 μg)/kg bw per day) for up to 112 days had no effect on performance indicators or MetHb. There was also no statistical association between nitrite dosing and vitamin A.

Reference point for nitrate in bovines

The CONTAM Panel identified a study by Benu et al. (2016) as the critical study. The authors measured the MetHb content of blood in 12 *Bos indicus* steers. Animals were exposed to a daily nitrate dose of 0, 30, 40 or 50 g NO_3^-/day for 7 days. Blood samples were performed every 2 h starting at 06.00 h and continued for a period of 7 days and the MetHb content in the blood of steers increased dose-dependently with nitrate dose.

The CONTAM Panel used the maximum MetHb content measured at each of the exposure days 2, 4, 5, 6 and 7, as relevant dose metric for BMD calculations (data used are listed in Appendix B). The CONTAM Panel chose a benchmark response (BMR) of 10% (% MetHb of total Hb), considering that hypoxia may occur at MetHb levels exceeding this value (Benu et al., 2016, 2018; Benu, 2017). The dose-response analysis and the calculation of the BMD and the $BMDL_{10}$, i.e. its 95% lower confidence limit was performed using BMDS Version 2.7.0.4 (US EPA, 2017). This is because it is a more transparent way to define the BMR as a specific response value (such as the 10% of MetHb). Modelling was performed as described in Appendix B.I.

The calculation of the BMD, the $BMDL_{10}$ and the BMDU at each sampling day is presented in Appendix B.I. The lowest $BMDL_{10}$ of 20 (rounded) g NO_3^-/day was chosen, corresponding to the exponential model 5 at day 2. Therefore, the CONTAM Panel chose a $BMDL_{10}$ of 20 g NO_3^-/day, corresponding to 64 mg NO_3^-/kg bw per day using the average weight of 317.8 kg reported in the study by Benu et al. (2016) as the reference point to be used in the hazard assessment of nitrate.

Following the comments received during the public consultation (EFSA, 2020), the Panel also performed BMD analysis with the PROAST web tool, by considering the cut-off value of 10% increase of MetHb (see above) as a percentage change compared to background response (as defined in EFSA Scientific Committee, 2017). This was done by back-calculating a threshold level of MetHb from the definition of continuous BMR given in PROAST. This corresponds approximately to a BMR of 450%.

This additional analysis is now presented in Appendix B.II and provides BMDL values similar to those derived using BMDS (overall model averaging BMDL-BMDU interval of 25–31 (rounded) mg nitrate per day).

Reference point for nitrite in bovines

In its previous Opinion (EFSA, 2009), the CONTAM Panel reported an NOAEL for nitrite in cattle of 3.3 mg/kg bw per day. This value was calculated by first extrapolating from nitrate LD_{50} to nitrite LD_{50}, using a relative potency factor of 10. The nitrite LD_{50} was then converted to LOAEL using a factor of 10. Finally, the nitrite LOAEL was extrapolated to an NOAEL using a factor of 3. However, the CONTAM Panel notes that acute toxicity values for cattle like LD_{50} are not currently used for deriving reference points and that there is little scientific ground for deriving a relative potency factor of nitrite relative to nitrate.

A non-peer-reviewed publication (Kozianowski et al., 2018) describes a study on MetHb formation in response to nitrite exposure. The study was performed in four Holstein cows (initial bw 725 $\pm$ 28 kg) fitted with a rumen cannula using a double 2 $\times$ 2 Latin square as experimental design. The animals were gradually introduced to increasing dietary levels of nitrate (14 days, 40 mg NO_2^- and 1,400 mg NO_3^-/day) followed by a 7-day period of daily administration of 7.5 mg NO_2^-/kg bw per day, directly into the rumen. Ruminal nitrite concentration decreased rapidly after dosing (–80% in 1 h) in treated cows, but no substantial differences in the rumen nitrate content were found. No substantial treatment-related differences were observed in rumen ammonia and blood MetHb content, the latter being stable over time (range 0.9–1.3%). Blood nitrite was not determined. The authors concluded that 7.5 mg NO_2^-/kg bw per day may be regarded as the NOAEL for nitrite in ruminants. The CONTAM Panel noted a number of weaknesses in the study, including the very low number of experimental animals (4) and the inclusion of a single dose level. In addition, while the rapid decrease in nitrite ruminal content suggests an increase in nitrite reductase as a possible consequence of the adaptation period, the very low MetHb levels are difficult to interpret in the absence of matched blood nitrite values.

Based on the above deficiencies, no NOAEL could be derived from this study.

Since the CONTAM Panel could not identify any other studies which could be used to determine an NOAEL or benchmark dose, the Panel is not in the position to propose a reference point for nitrite in bovines.

Ovines and caprines

Lewis (1951) injected 0, 12, 17.5, 22.5 and 25 g of sodium nitrate in the rumen of Oxford Down x Halfbred 3-year-old wethers (60 kg bw) (corresponding to approximately 0, 145, 220, 270, 300 mg NO_3^- /kg bw per day) 16 h after they had ingested their daily ration. The sheep had not been previously accustomed to nitrate in their feed. Rumen fluid concentrations of nitrate, nitrite, ammonia and MetHb were measured. In this study, the mean MetHb content of the control animals was about 2%. In animals fed 12 g nitrate per day, daily peak MetHb values were below 10% MetHb. The dose rates of 17.5, 22.5 and 25 g of nitrate per day demonstrated peak MetHb concentrations that increased from 15, 28 and 60%. It can be estimated that 120 mg NO_3^-/kg bw per day will not increase the MetHb levels above 10%.

No new studies were identified involving direct administration of nitrite in multiples doses to sheep without prior adaptation. In the previous CONTAM Opinion on nitrite (EFSA, 2009), a study by Trif et al. (1993) was used to set a reference point for nitrite in sheep. In this study, two groups of five ewes were administered 15 mg and 25 mg of sodium nitrite/kg bw per day for 60 days corresponding to 10 and 17 mg NO_2^-/kg bw per day in two separate experiments. The experimental conditions were not described. The MetHb content of the blood increased to a maximum of 4.5 and 7%, respectively, without clinical signs. From the same study, the CONTAM Panel reported in its previous Opinion an NOAEL for nitrite to sheep of 10 mg NO_2^-/kg bw per day. However, as the experimental conditions in Trif et al. (1993) were unclear, this study could no longer be considered for setting a reference point for nitrite.

Reference point for nitrate and nitrite in ovines and caprines

There is insufficient information to set reference point for nitrate for ovines and caprines. However, the data available suggest that they are not more sensitive than bovines. Therefore, the CONTAM Panel considers that the reference point selected for cattle can also apply to ovines and caprines.

Because of a lack of appropriate studies, the CONTAM Panel is not in the position to propose a reference point for nitrite in ovines and caprines.

3.1.3.2. Adverse effects in non-ruminants

Further details of the studies described below including animal body weights and feed/water intake of non-critical studies are summarised in Appendix A, Table A.2.

Pigs

The generation of MetHb from the reaction between nitrite and oxyhaemoglobin is also the mediator of the adverse effects following exposure to nitrate and nitrite in pigs (EFSA, 2009). The resulting depression of aerobic metabolism is associated with many clinical signs, such as restlessness, polyuria, vomiting, diarrhoea, dyspnoea, cyanosis, severe breathing difficulties, paralysis, unconsciousness and death (Wendt, 1985).

In a study by Wood et al. (1967), two experiments of 84 days were conducted in pigs (bw ~ 51.2 kg), one using potassium nitrate and the other using potassium nitrite. In the first experiment, nitrate was added to drinking water for pigs (daily WI calculated to 11.5 L) at levels of 0, 460, 920 and 1,840 mg NO_3^-/L drinking water. No differences were observed in animal performance or blood parameters at any treatment level. The highest exposure level of 1,840 mg NO_3^-/L water corresponds to 410 mg NO_3^-/kg bw per day. In the second experiment of Wood et al. (1967), nitrite was added to drinking water for pigs at levels of 0, 108, 216 and 432 mg NO_2^-/L drinking water. No differences were observed in feed gain, haemoglobin and haematocrit levels at all treatments. Body weight gain decreased with the lowest and highest nitrite levels (108, and 432 mg NO_2^-/L) against the control, although no changes in body weight gain were observed in the middle exposure level. However, MetHb levels increased with the highest nitrite level (432 mg NO_2^-/L corresponding to 94 mg NO_2^-/kg bw per day) against the control by 5.3-fold.

Recently, van den Bosch et al. (2019a,b) reported that the use of calcium nitrate in the maternal diet of sows up to 1,500 mg NO_3^-/kg of diet corresponding to 19 mg NO_3^-/kg bw from day 108 of gestation until 5 days after farrowing without any adverse effects.

In an experiment conducted by Seerley et al. (1965), sodium nitrite was added to drinking water for pigs at levels of 0, 82.1, 164.2 and 328.5 mg NO_2^-/L drinking water, corresponding to 0, 6.2, 13 and 25 mg NO_2^-/kg bw per day for 69 days. No differences were observed in animal performance. However, MetHb levels increased with 164.2 and 328.5 mg NO_2-/L by 3.77-17-fold compared to 0 and 82.1 mg NO_2^-/L.

London et al. (1967) reported that, in an acute toxicosis trial, single oral doses (via stomach tube) of KNO_2 up to 20 mg NO_2^-/kg bw did not cause any clinical signs to pigs (8–10 weeks of age), while single oral doses of 40 mg NO_2^- and 60–65 mg NO_2^-/kg bw caused mild (restlessness, frequent urination, vomition and detectable dyspnoea) and moderate (preceded by mild signs and consisted of more pronounced dyspnoea and detectable, cyanosis within 90 min) clinical signs, respectively. Single oral doses of 70, 75, 80 and 100 mg NO_2^-/kg bw caused severe clinical signs (preceded by moderate signs and consisted of marked dyspnoea and cyanosis followed by coma) and death of all treated pigs. In a chronic toxicosis trial, repeated oral doses 0, 10, 20, 30, 40, 60 mg NO_2^-/kg bw per day up to 124 days in drinking water were administrated. Pigs fed with 40 mg NO_2^-/kg bw per day did not show clinical signs, with MetHb levels ranged from 1.1 to 12.3% in their blood, while those fed the highest amount of nitrite 60 mg NO_2^-/kg bw per day frequently developed dyspnoea and cyanosis, with these signs lasting for 1–2 h, after drinking the NO_2^- containing water. From the study of London et al. (1967), and specifically the acute toxicosis trial, as the pigs use were younger, and therefore, more sensitive than the pigs used in the chronic toxicosis trial, the value of 20 mg NO_2^-/kg bw can be considered as the NOAEL for pigs and the value of 40 mg NO_2^-/kg bw can be considered as the LOAEL for pigs.

Mortality of pigs from nitrite has been mentioned at doses of 20 mg NO_2^-/kg bw and higher (Wendt, 1985; Muirhead and Alexander et al., 1997; Beilage et al., 2002; Vyt et al., 2005). However, these doses were mentioned in books or calculated in field reports and cannot be confirmed.

In addition, Shapiro et al. (2016) reported that pigs that consumed a single oral lethal dose of 920 mg NO_2^-/kg bw of paste bait died on average 1 h after ingesting the toxic bait.

Reference point for nitrate and nitrite in pigs

A dose of nitrate of 410 mg/kg bw per day can be considered as the NOAEL for pigs, while an LOAEL has not been identified based on the study of Wood et al. (1967). In addition, a single oral dose of nitrite of 20 mg /kg bw per day, in which no clinical signs are observed, can be identified as the NOAEL and a dose of nitrite of 40 mg NO_2^-/kg bw per day as the LOAEL based on the study of London et al. (1967).

Rabbits

Dollahite and Rowe (1974) experimentally induced acute toxicity in New Zealand White rabbits with nitrite or nitrate delivered as single oral dose by gavage. The LD_{50} to nitrite was determined to 124 mg NO_2^-/kg bw when given as $NaNO_2$ and 108 mg NO_2^-/kg bw when administered as KNO_2. The LD_{50} to nitrate was 1,955 mg from $NaNO_3$ and 1,166 mg from KNO_3.

In a 45-week experiment, male rabbits were fed diets supplemented with nitrate at 0, 255 and 510 mg NO_3^-/L water (Attia et al., 2013, 2018), corresponding to 0, 42 and 94 mg NO_3^-/kg bw per day, respectively. Nitrate at 510 mg NO_3^-/L water negatively affected reproductive performance, semen quality, blood haematological and biochemical parameters (Attia et al., 2013) and digestive, liver and kidney functions (Attia et al., 2018). Attia et al. (2013, 2018) concluded that rabbits may tolerate up to 255 mg NO_3^-/L water (42 mg NO_3^-/kg bw per day).

In an experiment conducted by Sharma et al. (2013a, b), rabbits were given water containing nitrate at 32.8, 72.9, 145.8, 291.7 and 364.7 mg NO_3^-/L, for 120 days, corresponding to approximately 7.3, 16, 33, 66 and 82 mg NO_3^-/kg bw per day, respectively (see Appendix A, Table A.2). There was no unexposed control, but the group exposed to 7.3 mg NO_2^-/kg bw per day was assigned as 'control'. The animals in all groups except the 'control' were found to be lethargic during the course of the experiment with lethargia first appearing in the high-dose group after 60 days of treatment (Sharma et al., 2013a). Cyanosis was reported for rabbits exposed to nitrate doses of 33 mg NO_3^-/kg bw per day and higher. Rabbits in all groups, including the 'control', showed increases in heart and respiration rates during the course of the 120-day experiment, and these effects appeared to be dose-related (no statistical analysis). Histopathological changes in the liver and the gastrointestinal tract were reported, but no conclusion could be drawn from the data presented. The two reports by Sharma et al. (2013a,b) would indicate that adverse effects of oral nitrate exposure appeared at a dose of 16 mg NO_3^-/kg bw per day and that no adverse effects were observed at the 'control' group exposed to 7.3 mg NO_3^-/kg bw per day. However, considering the low number of biological replicates (n = 2), that there was no unexposed control in this experiment, and that increased heart and ventilation rates were observed in all groups, the CONTAM Panel could not use this study to establish a reference point for rabbits.

In an experiment conducted by Akasha et al. (2015), rabbits were given water containing nitrate (not specified as salt or ion) at levels of 9 (background control), 64, 78.2, 144 and 200 mg NO_3^- /L drinking water for 14 weeks. In all treatments receiving nitrate, animal performance (body weight, feed intake and water intake) and T3 and T4 blood levels were negatively affected.

In addition, in an experiment conducted by Rashid et al. (2019), sodium nitrate was added to feed for rabbits at levels to 290 mg NO_3^-/kg bw per day, without or with various aqueous garlic extract doses for 40 days. The nitrate supplementation increased blood nitrite levels within 10 days after the experiment started until the end of the experimental period, and adversely affected the blood parameters (increase of ALT, AST, ALP, uric acid, urea, creatinine, blood glucose, serum cholesterol and decrease of albumin, total proteins, bilirubin), while no gross lesions were observed to any treatment group in various organs (liver, kidneys, lungs, brain, heart, intestine, pancreas). Aqueous garlic extract supplementation resulted in the reduction of such effects.

Overall, the CONTAM Panel could not identify any studies to establish reference points for nitrate and nitrite in rabbits.

Poultry

In an experiment reported by Adams et al. (1969), sodium nitrate was added to drinking water for turkey poults at levels of 0, 3,325, 3,990 and 4,655 mg $NaNO_3$/L for 49 days. All turkeys receiving nitrate suffered significantly greater mortality than the control group (8% vs. 17, 40, and 60%, respectively) at the end of the experimental period. The value of 3,325 mg $NaNO_3$/L water corresponds to 280 mg NO_3^-/kg bw per day. Three other experiments with turkey poults, reported by Adams et al. (1969), were not considered due to high mortality (that exceeded 14.5%) in the groups not receiving nitrate, or due to uncertainty to the causes of death.

Also, in quails, Adams (1974) conducted a series of three experiments in which various levels of sodium nitrate were added continuously to the water of quail (from 1,925 to 4,332 mg NO_3^-/L drinking water) from 1 to 7 days old. In all quail receiving nitrate mortality increased when compared to the control group. In a fourth experiment with quails from 1 to 105 days old, Adams (1974) reported that quails receiving sodium nitrate at levels 0, 481, 962, 1,925, 2,888 and 3,851 mg NO_3^-/L of drinking water showed mortality of 0, 4.4, 8.9, 15, 27 and 100%, respectively.

In an experiment reported by Grizzle et al. (1996), 12 groups of chickens for fattening were used in a 4×3 experimental design, with increasing levels of sodium nitrate in the drinking water at levels of 8.41, 12.05, 15.72 and 23.00 mg NO_3^-/L, and three water pH values (5.75, 6.25 and 6.75). The experiment lasted 42 days. The overall results suggest that final body weight and relative thymus weight decreased with 12.05, 15.72 and 23.00 mg NO_3^-/L of drinking water compared to the control group subjected to 8.41 mg NO_3^- /L of drinking water, equivalent to a dose of 1.1 mg/kg bw per day. Considering the lack of unexposed control in this experiment and the limited number of tested parameters, the CONTAM Panel could not use this study to establish a reference point for poultry.

Recently, the exposure of broiler chickens via drinking water to sodium nitrate (20.0 mg NO_3^-/L water, corresponding to 2.7 mg NO_3^-/kg bw per day), negatively affected body weight gain (1,588 vs. 1,494 g) during the finisher period (25–42 days of age) and feed to gain ratio (1.77 vs. 1.85) during the overall period (1–42 days of age) of the experiment compared to the 'control' of 3.9 mg NO_3^-/L water (0.5 NO_3^-/kg bw per day) (Akhavast and Daneshyar, 2017). Nitrate also decreased blood uric acid, total antioxidant capacity and blood pO_2 and increased blood pCO_2. Considering that only one dose of nitrate was included in this study, as positive control to test the antioxidant effects of rosemary extracts, and the lack of an unexposed control in this experiment the CONTAM Panel could not use this study to establish a reference point for poultry.

In two experiments conducted by Sell and Roberts (1963), potassium nitrite was added to feed for cockerels at levels equivalent to 0, and 2,165 mg NO_2^- /kg feed, without or with various vitamin A doses for 28 days. When comparing the two treatments that were not supplemented with vitamin A, the performance of cockerels was adversely affected by the nitrite supplementation, while a higher death rate was also recorded (Exp. 1: 10 vs. 44%; Exp. 2: 7 vs. 33%). Vitamin A supplementation resulted in the reduction of such effects. The value of 2,165 mg NO_2^-/kg feed corresponds to 130 mg NO_2^-/kg bw per day.

Stoewsand (1970) reported depressed growth rates, a decrease in haemoglobin and an increase in MetHb levels, when quail 15 weeks of age were fed either 0 or 5,000 mg NO_2^- (as $NaNO_2$)/kg feed for 1 week.

Diaz et al. (1995) fed chickens (in a 35-day exp.) and turkeys (in a 14-day exp.) with 0, 200, 400, 800, 1,200, 1,600 mg NO_2^- /kg feed and, with the 1,600 mg NO_2^-/kg feed dose, reported a reduction in growth rate (total bw: chickens 1,600 vs. 1,403 g, turkeys 218 vs. 188 g), a decrease in haemoglobin and an increase in MetHb levels. For chickens, the value of 1,600 mg NO_2 /kg feed corresponds to 96 mg NO_2^- /kg bw per day.

In an experiment conducted by Bilal and Can Kutay (2002), sodium nitrite was added to feed for chickens at levels equivalent to 0, and 667 mg NO_2^- /kg feed, without or with various vit. E inclusion levels. Each experiment used 60 chickens/treatment (20 chickens/replicate pen, 3 replicate pens/ treatment) and lasted 35 days. When comparing the two treatments that were not supplemented with vit. E, the nitrite supplementation adversely affected the performance of chickens (total bw 1,714 vs. 1,597 g), as well as blood parameters (decrease of haemoglobin 17.90 vs. 15.04 g/100 mL, increase of MetHb 1.04 vs. 1.15 % of total pigment), while a higher death rate was also recorded (0 vs. 10%). The value of 667 mg NO_2^- /kg feed corresponds to 30 mg NO_2^- /kg bw per day.

Shapiro et al. (2017) assessed acute toxicity of sodium nitrite ($NaNO_2$) in chickens (*Gallus gallus domesticus*) and domestic mallard ducks (*Anas platyrhynchos domestica*) by oral gavage and in free-feeding trials with chickens, domestic mallard ducks, pigeons (*Columba livia f. domestica*), budgerigars (*Melopsittacus undulates*) and wētā (Family: Rhaphidophoridae). The authors reported an LD_{50} value for $NaNO_2$ in solution of approximately 45.5 mg NO_2^-/kg bw for both chickens and ducks by oral gavage. In feeding trials, four out of six chickens consumed a lethal dose of toxic paste bait and the authors calculated an LD_{50} value for chickens of approximately 169.7 mg NO_2^-/kg bw.

In addition, in an experiment conducted by Strnad and Persin (1983), sodium nitrite and sodium nitrate were added to drinking water for pheasant chickens of 14 days of age at levels equivalent to 0, 15 mg NO_2^- /L (16 mg NO_2^-/kg bw) and 500 mg NO_3^-/L (580 mg NO_3^- /kg bw per day), respectively. The groups receiving 16 mg NO_2^-/kg bw per day and 580 mg NO_3^- /kg bw per day showed increase of MetHb content from 5.1 to 16.5% and 7.1%, respectively. The authors mentioned that with the nitrite supplementation, non-specific dystrophic changes in liver and kidneys, and villus oedema of the small intestine were also observed. The nitrate supplementation was said to cause hyperaemia of liver, kidneys and mucosa of the small intestine and multiplication of the eosinophilic granulocytes in the villus stroma. No quantitative description of the findings was provided.

In an experiment conducted by Atef et al. (1991), sodium nitrite and sodium nitrate were added to feed for cockerels of 2 months old at levels equivalent to 0, 1,133 mg NO_2^- /kg feed (68 mg NO_2^-/kg

bw per day) and 3,063 mg NO_3^-/kg feed (180 mg NO_3^-/kg bw per day), respectively. Each experiment used 15 chickens/treatment and lasted 28 days. The nitrite supplementation adversely affected the performance of cockerels as well as blood parameters (decrease of erythrocyte numbers 3.4 vs. 2.4 × 10^6/μL, increase of MetHb 1.1 vs. 25.6 % of total pigment, increase of glutamic–pyruvic transaminase 23 vs. 35 units/L, increase of creatinine 9 vs. 18 mg/L and increase of urea 200 vs. 300 mg/L). Moreover, the nitrate supplementation also adversely affected the performance of cockerels (total bw gain 210 vs. 180 g, decrease of the bursa relative weight 0.32 vs. 0.25 g/100 g bw), as well as blood parameters (decrease of erythrocyte numbers 3.4 vs. 2.9 × 10^6/μL, and increase of MetHb 1.1 vs. 8.0 % of total pigment).

Overall, reference points for nitrate and nitrite have not been identified for poultry due to lack of appropriate studies.

Horses

Horses do not convert nitrate to nitrite efficiently and, thus, are expected to be less susceptible to poisoning compared with ruminants. No specific data on nitrite toxicity are available.

Oruc et al. (2010) attributed the deaths of nine mares to acute nitrate poisoning (within 24 h) associated with ingestion of forage and alfalfa. Nitrate levels in pasture grass and alfalfa ranged from 400 to 9,923 mg NO_3^-/kg DM and 2,232–4,341 mg NO_3^-/kg DM, respectively, and total consumption of nitrate was estimated to approximately 80 g per animal or approximately 180 mg NO_3^-/kg bw per day.

Overall, in horses, reference points for both nitrite and nitrate have not been identified.

Fur animals

No specific data on nitrite and nitrate toxicity are available in fur animals.

Dogs and cats

Lehman (1958) reported that, when two dogs were fed with 14,588 mg NO_3^- /kg feed, no adverse effects in dog health or changes in blood parameters were observed. Considering the low number of biological replicates (n = 2) and the poor reporting, the CONTAM Panel could not use this study to establish a reference point in dogs.

An investigation was conducted of the effects of dietary nitrate on thyroid function in Beagle dogs (1 male and 6 females per group) and their offspring (Kelley et al., 1974). The dogs received 0, 218.8, 437.6 and 729.4 mg NO_3^-/L of drinking water under normal management and feeding conditions for 16 months. No clinical manifestation of hypothyroidism was observed in any of the adult or puppy Beagles. The value of 729.4 mg NO_3^-/L water corresponds to 460 mg NO_3^- /dog per day, and to 31 mg NO_3^- /kg bw per day.

Michalski (1963) reported induction of MetHb in dogs after oral exposure by gavage to sodium nitrite at doses ranging from 35 to 71 mg NO_2^-/kg bw for 3 days and reported a lethal dose of 53–71 mg NO_2^-/kg bw. When nitrite was administered in feed at a dose of 89 mg/kg bw, death was observed at different times (after 133–313 h). Considering the lack of unexposed control in this experiment and the limitations in the design of the study, the CONTAM Panel could not use this study to establish a reference point in dogs.

A 26-week toxicity assessment of sodium nitrite by intravenous administration in dogs was performed by Tepper et al. (2014). In this study, seven male and seven female Beagle dogs were used in four dose groups for a total of 56 dogs (approximately 15 months old, at the onset of the treatment); groups received 0, 4.67, 9.33, and 18.67 mg NO_2^-/kg bw per day. Given that cyanosis and MetHb were observed in dogs, primarily at the high dose group, the intravenous NOAEL was considered from the authors to be 9.33 mg NO_2^-/kg bw per day.

In cats, acute toxicosis with death due to extensive MetHb formation has been reported in three animals fed on a canned feed containing on average 1,900 mg NO_2^-/kg canned feed (Worth et al., 1997).

Lehman (1958) reported that no adverse effects were observed on growth performance and organ weight in one cat receiving an oral dose of 6.5 mg NO_2^-/kg bw per day, for a period of 105 days.

Overall, because of a lack of appropriate studies, the CONTAM Panel is not in the position to identify reference points for nitrate and nitrite in dogs and cats.

Fish

No studies were identified that assess toxicity of fish due to nitrate or nitrite in feed. Fish are sensitive to waterborne nitrate and nitrite. Indeed, elevated concentrations of nitrite and nitrate in the water are issues in aquaculture, and along with water ammonia concentrations, limit stocking density.

This is not directly caused by the content of nitrate and nitrite in fish feed but a consequence of almost all teleost fish excreting nitrogenous waste as ammonia which undergoes bacterial conversion to nitrite and further oxidation to nitrate, which accumulates in the water. An increase in nitrate and nitrite in feed would plausibly lead to a higher excretion of nitrogenous waste, but since their accumulation in the water depends on the rate of water exchange, it is impossible to determine upper limits for nitrate of nitrite in fish feeds.

Summary of reference points for nitrate and nitrite

Based on the literature reviewed above, with the acknowledged limitations, reference points for a range of livestock and companion animals are summarised in Table 4.

Table 4: Reference points of nitrate and nitrite for livestock and companion animals calculated from the literature

Species	Critical effect	Reference points mg ion/kg bw per day	References
NO_3^-			
Ruminants	Methaemoglobinaemia	64 ($BMDL_{10}$)	Benu et al. (2016)
Pigs	Performance and blood parameters	410 (NOAEL)	Wood et al. (1967)
NO_2^-			
Pigs	Clinical signs	20 (NOAEL)	London et al. (1967)

3.1.3.3. Formation of N-Nitroso compounds (N-nitrosamines) related to nitrate/nitrite in feed and their transfer to animal products

N-nitrosamines are organic compounds formed upon the reaction of nitric oxide, as a nitrosating agent generated from nitrite, with secondary amines. This reaction could occur outside or inside the body. In the former case, secondary amines are widely distributed in food and feed (e.g. dimethylamine, diethylamine) or may be a component of amino acids (e.g. proline, hydroxyproline, carnitine, citrulline). N-nitrosamines can also be formed *in vivo*, especially under acidic conditions (stomach); the reaction depends mainly on the amount of nitrite ingested or formed from nitrate, the amount of nitrosatable substances available, and the rate of nitrosation at a specific pH. N-nitrosamines are of toxicological relevance, because some of them could be genotoxic and carcinogenic (SCCS, 2012; EFSA ANS Panel, 2017a), both for the animals and for humans who consume animal products. The most important dietary N-nitrosamines are low molecular weight and cyclic N-nitrosamines such as N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopyrrolidine and N-nitrosopiperidine (EFSA ANS Panel, 2017a).

N-nitrosamines may be formed in feed. Historically, nitrite has been used as a feed preservative for fishmeal (no longer permitted nowadays). Sodium nitrite, when used to preserve fishmeal for feed, has been reported to react with secondary amines present in stale fish (particularly dimethylamine and trimethylamine) during the processing and storage of the feed and to produce NDMA (30–100 mg/kg, as cited in Sen et al., 1972). Toxic outbreaks have been documented in the 60s of the last century in cattle, sheep and fur animals consuming N-nitrosamine-contaminated fishmeal (reviewed by Bruning-Fann and Kaneene, 1993); in all cases affected animals exhibited signs of liver damage (Koppang, 1974), the target organ for NDMA (SCCS, 2012).

Very little is known concerning the preformed N-nitrosamine content of feedstuffs. In one of the few studies (a conference paper), Juszkiewicz et al. (1980) collected commercial mixed feeds and protein concentrates samples (n = 495) from nearly 100 feedmills in Poland, including waste-containing feeds, fish meal for the processing of mixed feeds for animals, samples of krill meal, grain and grass hay. All samples were first analysed for nitrate/nitrite content (some also for easily nitrosatable dimethylamine (DMA)); only krill meal samples and samples containing the highest nitrate/nitrite levels (n = 171) were then analysed for volatile N-nitrosamines. Over 62% of the total samples contained nitrate in concentrations ranging from 1 to 1,020 mg/kg, the highest values being detected in waste-containing feeds. In contrast, only 6% contained 1–15 mg/kg nitrite. Forty percent of the 171 selected samples having the highest levels of nitrate, nitrite and amines contained the volatile NDMA (range 0.003–0.417 mg/kg) No statistical correlation was found between concentration of nitrate/nitrite and N-nitrosamines. Remarkably, no measurable amount of nitrate, nitrite and NDMA were detected in krill meal, which showed instead high DMA concentrations (110–1,765 mg/kg). In line with the presence of DMA and other nitrosatable amines, the in vitro incubation of fishmeal and krill meal samples with

sodium nitrite at 37°C, pH 3.4 resulted in the formation of NDMA in the range 0.7–2.3 mg/kg, pointing to the 'endogenous' formation of N-nitrosamines. Van Broekhoven and Davies (1985) analysed 20 different silage samples from grass and maize, grown in fields dressed with various amounts of nitrogen fertiliser and manure, for the presence of volatile N-nitrosamines. NDMA (LOD 0.2 μg/kg) was the only detected N-nitrosamine with concentrations ranging from 0.2 to 4 μg/kg fresh weight (fw).

The endogenous formation of N-nitrosamines in ruminants and their transfer to animal products has been mostly addressed in a series of conference papers. Juszkiewicz et al. (1975) carried out experiments on milking goats. The administration of a single oral dose of sodium nitrite (80 mg/kg bw) or fresh kale (3% potassium nitrate dry weight (dw)) together with 100 mg/kg bw dimethylamine hydrochloride (DMA.HCl) or 200 mg/kg bw diethylamine hydrochloride did not cause the formation of measurable amounts of N-nitrosamines either in blood or in milk 6 h after dosing. In a companion trial, the dosages were increased to 150 mg sodium nitrite kg/bw and 300 mg DMA.HCl per kg bw, and 15 g sugar/kg bw was added to induce acidosis and to lower rumen pH to 4.0–5.0. Following the adoption of the new protocol, introducing more favourable conditions for N-nitrosamine formation, only very limited blood concentrations of N-nitrosamines (30 μg/kg) could be measured after 2 h, while trace amounts were detected in milk 2 h but not 4 h after dosing.

The *in vivo* formation of N-nitrosamines under more physiological conditions was studied in cows. Only traces (up to 0.3–0.45 μg/kg) of volatile N-nitrosamines (NDMA, NDEA and N-nitrosopyrrolidine) were determined in rumen samples from a fistulated cow 1 h after a single administration of 260 mg potassium nitrate/kg bw; already 2.5 h after dosing, N-nitrosamines reached undetectable levels (Van Broekhoven and Stephany, 1978). In another experiment, very low levels of NDMA (0.1–0.4 μg/kg, LOD 0.1 μg/kg) were found in rumen fluid specimens collected from two non-lactating cows for up to 2.75 h after the administration of 120 mg/kg bw of nitrate as potassium nitrate (Van Broekhoven and Davies, 1981). Another trial (Van Broekhoven et al., 1984) was designed in dairy cows to assess the fate and the possible milk transfer of N-nitrosamines already present in feed (N-nitrosoproline,N-PRO) or formed *in vivo* after the dietary exposure to nitrate and an easily nitrosable amino acid (proline). Cows were fed rations with variable content in nitrate (0.05–4.29 g $N\text{-}NO_3^-$/kg DM), free proline (0.89–15.27 g/kg fw) and N-PRO (13–377 μg/kg fw) for 3 days and milk, urine, faeces and rumen fluid (fistula) samples were collected for the subsequent 3 days. No N-PRO was found in the milk or there was evidence of newly formed N-PRO in the rumen. N-PRO already present in the feed was recovered nearly quantitatively in the urine and faeces, in almost equal percentages. The lack of endogenous formation of N-PRO was confirmed by a further experiment on cows adopting a similar protocol (Van Broekhoven et al., 1989).

In the cited study of Hegarty et al. (2016) (see Section 3.1.1.2), meat samples from 432 slaughtered composite-meat breed steers, fed with diets containing different levels of urea or calcium nitrate (approximately corresponding to 11 or 20 g N-NO3-/kg) for 102 days were tested for nitrosamines. The raw or cooked samples did not contain detectable levels of the tested nitrosamines (LOD in brackets), namely N-nitrosodiethanolamine (< 0.02 mg/kg), N-DMA (< 0.01 mg/kg), N-nitrosomethylethylamine, N-nitrosopyrrolidine (< 0.01 mg/kg), N-nitrosopiperidine (< 0.02 mg/kg), N-nitrosomorpholine (< 0.2 mg/kg), N-nitrosodi-n-propylamine (< 0.01 mg/kg), N-nitroso-di-n-butylamine (< 0.1 mg/kg) or N-nitrosodiphenylamine (< 0.1 mg/kg).

As regards monogastric species, experimental studies (Lintas et al., 1982) were performed in dogs with direct administration (gastric fistula) of 30–120 mg sodium nitrite together with 50 mg dimethylamine (DMA). At gastric pH values between 3 and 5, there was a rapid increase of NDMA to the highest levels (up to 50 μg/kg of gastric content) in less than 10 min followed by a complete disappearance within 30 min, pointing to NDMA absorption through the gastric mucosa. The rate of DMA nitrosation was very low at stomach pHs > 5 and was hampered by the addition of ascorbic acid.

The CONTAM Panel noted that although there was no statistical correlation between concentrations of nitrate, nitrite and N-nitrosamines, there might be formation of N-nitrosamines in feed, in particular in fish meal due to the presence of nitrate/nitrite and secondary amines. However, no recent intoxication of animals has been reported by fishmeal, due probably to the setting of maximum limits of nitrite in fishmeal (30 mg/kg). Based on a limited data set, the N-nitrosamine contamination of different feedstuffs seems to be very low.

A limited number of old studies in a few animal species showed a very low in vivo formation of N-nitrosamines due to the experimental administration of high amounts of both nitrite and secondary amines, i.e. under feeding conditions unlikely to be met under normal feeding regimes. In addition, few experiments indicate that the milk transfer of N-nitrosamines either endogenously formed or already present in feed is negligible. Finally, the dietary supplementation of beef cattle with nitrate at

dosages used to mitigate methane emissions did not result in the accumulation of measurable amounts of N-nitrosamines in meat.

The CONTAM Panel considered that although N-nitrosamines could be produced endogenously, the evidence to assess the risk is very limited and that there is no information to link this endogenous production of N-nitrosamines with adverse effects in farmed and companion animals. From the scarce studies available, the transfer of N-nitrosamines to meat or milk in ruminants seems negligible.

3.2. Feed occurrence data

3.2.1. Previously reported feed occurrence data in the open literature

Nitrate are essential for plant growth and are widely present in all parts of the plant, but predominantly in the leaves and stems. Plants normally take up nitrogen from the soil in the form of nitrate, but when growth is normal little nitrate accumulates in plants because it is rapidly converted to plant amino acids and protein.

However, more than 80 forage species have been reported to accumulate nitrate at levels that have resulted in adverse health effects in livestock consuming them (Clarke and Clarke, 1975; Hall, 2018). The majority of these are weeds, not grown as feeds for livestock, but nitrite poisoning has been reported as a result of accidental consumption. Several common agricultural crops, including oats, barley, maize (corn), soybean, sunflower and wheat are also known to accumulate nitrate to levels that may be toxic. With the exception of maize, consumption of the growing crop by livestock is usually accidental. Among the cereals, oats have the propensity to accumulate higher levels of nitrate in the grains than other cereal crops (Sidhu et al., 2011). This is reflected in higher levels of nitrate in oat grains than other cereal grains in the database (Annex III, Table III.6)

Several feed crops, grown mainly as feeds for ruminants and horses,[20] may, under certain conditions, accumulate nitrate to levels that result in nitrite toxicity. These crops include commonly grown grasses (*Lolium spp.*), sugar beet (*Beta vulgaris*), members of the *Brassica* family, including cabbages, kale, forage rape, stubble turnips and swedes and clovers (*Melilotus officinalis*). High nitrate levels in these crops are directly correlated with levels of nitrogen fertiliser applied (Lovett et al., 2004), although other varietal, environmental and management factors may also influence the concentrations of nitrate in these plants. High levels of nitrogen application have been widely reported to result in elevated nitrate levels. In addition, moisture stress, decreased light (cloudiness, short day length) and low temperatures have been reported to result in higher levels of nitrate in plant tissue due to a reduction in the rate of amino acid synthesis from nitrate transported from the roots under these conditions (Paul and Myers, 1971; Laine et al., 1994).

Forages, including grass (*Gramineae spp*.), maize (*Zea mays*), lucerne (*Medicago sativa*) and clover (*Trifolium spp*.), either fresh or conserved as hay or silage, are major ingredients in diets of ruminant livestock and horses, but insufficient data on nitrate and nitrite levels in these feeds were obtained as part of the EFSA call for data. However, nitrate analysis is routinely part of forage analyses throughout the EU; although the results of these analyses are not widely publicised, for this Opinion data for conserved forages sampled and analysed in the UK and the Netherlands were obtained and used. Details are given below:

- *Grass silage:* The annual mean nitrate contents of UK grass silages analysed between 2015 and 2019 ranged from 2.1 (2019) to 3.7 (2017), with a mean 5-year average of 2.6 g NO_3^- /kg DM.[21] This is similar to levels reported for the Netherlands, which ranged from 1.6 (2015) to 3.9 (2018) g/kg DM, with an overall mean of 2.6 g/kg DM.[22]
- *Maize silage:* Measured levels ranging from 1.4 to 2.3 g NO_3^- /kg DM were reported by Van den Top (2005), from samples obtained between 1997 and 2002. Nitrate levels ranged from 0.0 to 2.3 g/kg DM, with a mean of 0.88 mg/kg DM, and this value was used to estimate exposure on maize silage-based diets.

There is also limited information in the published literature on levels of nitrate in cereal grains or oilseed meals. Although nitrate levels in grains are generally lower than in other parts of the plant (Wu and McDonald, 1976), levels are variable. For example, concentrations in wheat grains varied between 0.4 and 11 mg/kg and were influenced by both the plant variety and the growing conditions

[20] Horses are less sensitive to nitrate toxicity since they are hindgut ferments and do not have a rumen.
[21] Source: Daniel Robinson, Eurofins Agrotesting UK Ltd., Wolverhampton, UK (personal communication).
[22] Eurofins: https://www.melkvee.nl/site/assets/files/0/84/070/graskuilned2018_1007voorjaar.pdf

(McNamara et al., 1971). For soybean seeds (i.e. before oil extraction), McNamara et al. (1971) reported a range of 8.6–22.9 mg/kg DM, although it is unclear how this would be affected by subsequent oil extraction and processing to produce the meal. Bhatty et al. (1973) detected no nitrate in soybean meal.[23]

Nitrite are not present in soils to any significant extent and not taken up by plants (Archer, 1985). A review of the scientific literature by EFSA (2009) reported that nitrite analysis is rarely part of routine analyses of feeds, and therefore, few data are available. No data were identified for levels of nitrite in fresh or conserved forages.

3.2.2. Feed occurrence data submitted to EFSA

By the end of October 2019, an initial data set of 4,127 analytical results on nitrate and nitrite in feed was available for the present evaluation. The data were collected in 13 EU countries and three non-EU countries. In addition, a part of the analytical results referred to the EU countries or non-EEA countries as a place of collection without naming the country of origin. Overall, an important part of the analytical results was provided by industry, i.e. European Association of Sugar Manufacturers (CEFS EU Sugar), European Starch Industry Association (STARCH Europe) and The European Vegetable Oil and Protein Meal Industry Federation (FEDIOL). Limited number of data did not allow to evaluate the consistency between data submitted by the Member States (MSs) and the industry; therefore, the data were merged and considered as one data set. The major contributor of data was STARCH Europe which reported 33% of data covering different EU and non-EU countries followed by Slovakia (28% of data). Data were reported on samples collected between the years 2006 and 2019. However, in order to reflect the current contamination levels, only the most recent data were used in the assessment from 2010 onwards. The occurrence data for nitrate and nitrite on 4,127 feed samples is available on the EFSA Knowledge Junction Community on Zenodo.[24]

As mentioned in Section 3.2.1, additional information on nitrate levels in grass silage from the UK has been obtained from private agricultural commodity testing laboratories (Eurofins, 2020[22]; personal communication). Since the provided information on grass silage refers to the mean concentration in different years of sampling only, it is not possible to further describe these data. For maize silage, the data of Van den Top (2005) were used.

Analytical results were reported either as nitrate, nitrite, nitrate nitrogen or as sodium nitrite. Conversion factors were applied to convert these data into nitrate/nitrite ion; the nitrate nitrogen analytical results were divided by a factor of 0.23 and the sodium nitrite analytical results were multiplied by a factor of 0.65. No analytical data were reported as sodium nitrate, potassium nitrate or potassium nitrite.

The occurrence data were carefully evaluated, and a list of validation steps was applied before being used to estimate dietary exposure (see Annex III, Table III.1 for further details). The final data set comprised 1,542 analytical results on nitrate and 1,561 on nitrite.

All analytical data on nitrite with the LOQ above the ML of 15 mg/kg (expressed as sodium nitrite), laid down in the Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed, were excluded.

The LODs/LOQs of the remaining nitrate and nitrite data varied between laboratories, analytical methods and feed commodities. Lower median LODs/LOQs were reported for gas chromatographic (GC) methods as compared to other analytical methods, for 'Tubers, roots, and products derived thereof' (nitrate) and for 'Oil seeds, oil fruits, and products derived thereof' (nitrite), as compared to other feed categories (for further details, see Annex III, Table III.2). An evaluation of appropriateness of LODs/LOQs was performed by comparing the average LB with UB concentrations of the relevant feed commodities based on the typical expanded uncertainty associated with the analytical results, which in an ideal case is reported by the laboratory (Codex Alimentarius Commission, 2004). Although in most of the cases, uncertainty measurement is not reported by the data providers, all the analytical results possess an associated uncertainty that is highly influenced by the measured nominal concentration. As an example, typical expanded uncertainties when reporting nominal concentration between 10 and 100 mg/kg would be 15% (relevant for nitrate) or between 1 and 10 mg/kg would be 20% (relevant for nitrite). When the differences between average LB vs. UB estimations expressed in percentage of the LB ([UB – LB] × 100/LB) are lower than this specified percentage, no LOQ cut-offs

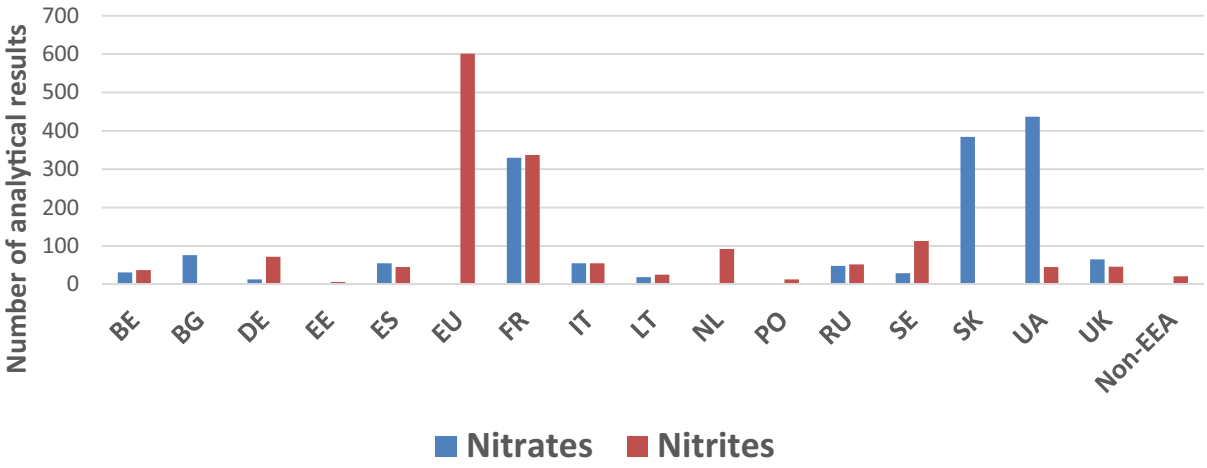
[23] Nitrate nitrogen was determined after treatment of the meals with the salicylic-sulfuric acid mixture described by Humphries (1956).
[24] https://doi.org/10.5281/zenodo.4061688

shall be applied on the data set (EFSA, 2018). Following this principle, no LOQ cut-offs were applied to the nitrate analytical results considered in the present assessment. Nevertheless, in the case of nitrite, the differences between average LB vs. UB estimations expressed in percentage was for several feed categories higher than 20%, due to limited number of nitrite data, it was not possible to identify an appropriate LOQ cut-off. Therefore, no LOQ cut-off was applied to nitrite data.

Approximately, 17% of the data were obtained for samples collected within the official monitoring programmes and 83% were collected by the industry within the private monitoring programmes. Regarding the sampling strategy, no analytical results were obtained by suspect sampling.

Results were reported on whole weight (91% of analytical results) or on 88% dry matter (9% of analytical results). For consistency, the latter ones were converted to values expressed on a whole-weight basis. The conversion was either based on the moisture content reported or, in case of lack of this information, a standard moisture percentage of 12% for dry feed commodities was assumed and applied.

The final data set contains a part of the analytical results which refers to the EU countries (19% out of all data) or non-European Economic Area (EEA) countries (< 1%) as a place of collection without specification of the country. The remaining analytical results were collected in 15 different European countries, including both EU and non-EU countries; most of them in France (21% and 22% of analytical results of nitrate and nitrite, respectively), Ukraine (28% of analytical results of nitrate) and Slovakia (25% of analytical results of nitrate). Figure 2 shows the distribution of analytical results of nitrate and nitrite across the place of collection. It should be noted that the origin of the samples was not always the same as the place of collection, i.e. the data set also contained samples originating from North and South America, Africa, Asia and Australia. The samples were collected between 2010 and 2019 (Figure 3).



BE: Belgium; BG: Bulgaria; DE: Germany; EE: Estonia; ES: Spain; EU: European Union; FR: France; IT: Italy; LT: Lithuania; NL: the Netherlands; PO: Poland; RU: Russia; SE: Sweden; SK: Slovakia; UA: Ukraine; UK: the United Kingdom; Non-EEA: non-European Economic Area countries.

Figure 2: Distribution of analytical results for nitrate and nitrite across the place of collection (after excluding non-qualifying data)

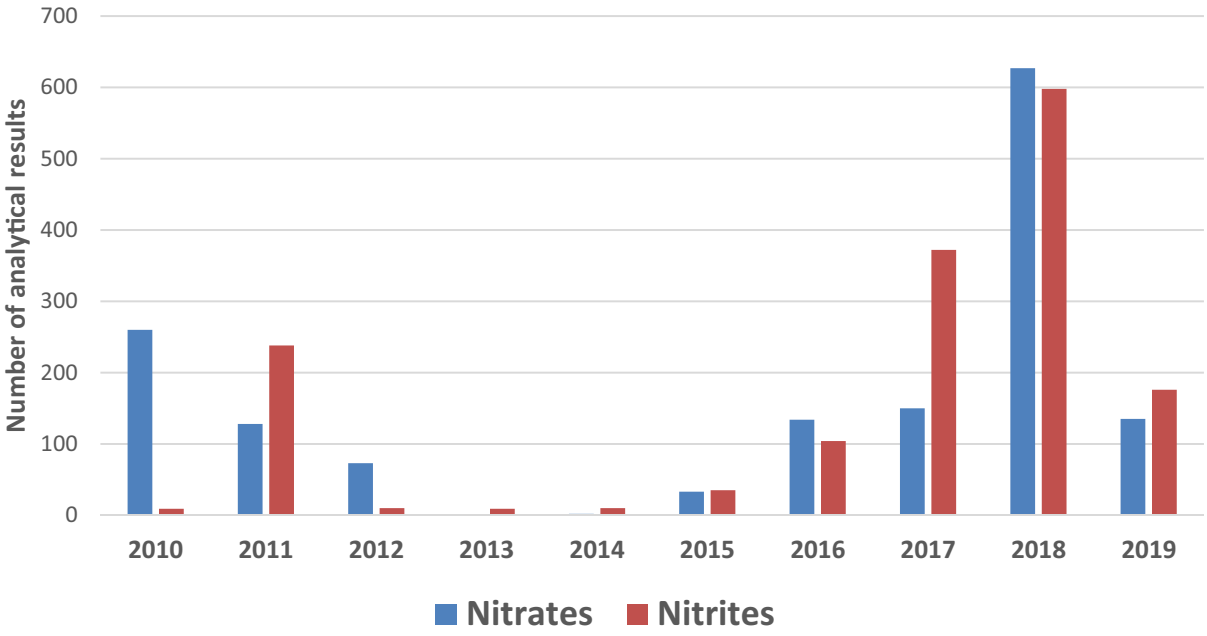


Figure 3: Distribution of analytical results for nitrate and nitrite by sampling year (after excluding non-qualifying data)

Table 5 summarises the number of analytical results and the percentage of left-censored data per substance and feed category at FoodEx level 1. Overall, 1,542 analytical results were available for nitrate and 1,561 analytical results for nitrite. Relatively low proportion of LCD was observed for nitrate (16%), whilst the data set of nitrite comprised more LCD data (43%).

Overall, the data set available for evaluation of nitrate and nitrite in feed was rather limited. Concerning nitrate, the most frequently analysed feed categories were 'cereal grains, their products and by-products' and 'oil seeds, oil fruits and products derived thereof'. For nitrite, a substantial number of analytical results was available for 'tubers, roots and products derived thereof' and 'cereal grains, their products and by-products'. Other feed categories were less covered and some of them (e.g. 'legume seeds and products derived thereof', 'other plants, algae and products derived thereof' etc.) comprised only limited number of data (Table 5).

Table 5: Distribution of analytical results per substance and feed category

FoodEx level 1 feed category	Nitrate		Nitrite	
	N	LCD	N	LCD
Cereal grains, their products and by-products	550	21%	590	74%
Fish, other aquatic animals and products derived thereof	–	–	26	96%
Miscellaneous	80	10%	63	67%
Oil seeds, oil fruits and products derived thereof	542	4%	127	51%
Legume seeds and products derived thereof	4	25%	–	–
Tubers, roots and products derived thereof	127	9%	718	13%
Forages and roughage, and products derived thereof	239	35%	1	100%
Other plants, algae and products derived thereof	–	–	4	50%
Compound feed	–	–	32	38%
Total	1,542	16%	1,561	43%

N: number of analytical results; LCD: left-censored data.

It should be noted that the data for 'Forages and roughage, and products derived thereof' were not used in the exposure assessment due to the imprecise categorisation (i.e. failure to differentiate between types of forage or whether they were fresh or conserved). Therefore, as mentioned in this opinion (Section 3.2.1), data for grass and maize silages were obtained and used for this purpose.

3.2.3. Analytical methods

As specified in Section 3.2.2 (for more details, see Annex III, Tables III.1), some of the nitrite analytical results obtained by analytical methods with LOQ higher than ML of 15 mg/kg (expressed as sodium nitrite) were not included in the final data set. Most results were obtained by the high-performance liquid chromatography (HPLC)-based methods (28% of nitrate analytical results and 50% of nitrite analytical data) and colorimetry, spectroscopy (spectrometry) and photometry' methods (23% of nitrate analytical results and 46% of nitrite analytical data). Small part of nitrate and nitrite data were analysed by the GC-based methods, ion exchange chromatography and immunochemical tests (ELISA). For the remaining samples, no information on the analytical method was reported.

The distribution of the LOQs for nitrate and nitrite across the FoodEx level 1 feed categories and across the analytical methods is summarised in Annex III, Table III.2. Only feed categories and analytical methods with ≥ 5 analytical results are shown). The highest median LOQs was observed for nitrate in 'Oil seeds, oil fruits, and products derived thereof' (16.2 mg/kg). Regarding the analytical methods, the median LOQs ranged from 0.5 mg/kg reported for GC-based analytical methods analysing nitrate to 10 mg/kg reported for ELISA and ICP-MS methods.

3.2.4. Occurrence data on feed by feed group

The presence of nitrates and nitrites in animal feed is principally the result of the active transport of nitrate from the roots to the vegetative parts of the plant. The extent and rate of this is influenced by many plant, management and climatic factors. It is beyond the scope of this Opinion to review these or discuss the relevance of these to levels of nitrates/nitrites reported in animal feeds.

Occurrence data of nitrate

Table 6 provides a summary of occurrence data on nitrate across the feed categories, including the number of results, percentage of left-censored data and statistical descriptors of the results (mean, median, 75th and 95th percentile). More detail on statistical description is reported in Annex III, Tables III.3–III.5. In addition, the summaries of occurrence data including the number of results, percentage of left-censored data and the mean LB and UB concentrations of nitrate according to the FoodEx food categories as used for exposure assessment are reported in Annex III, Table III.6.

The occurrence data on nitrate were available for six FoodEx level 1 feed categories with most analytical results available for 'Cereal grains, their products and by-products', in particular for wheat and maize. Nevertheless, for some other grains, e.g. barley, oats, only very limited number of analytical results was obtained. A substantial number of analytical results was available also for 'oil seeds, oil fruits and products derived thereof', in particular for sunflower seed. The highest nitrate mean concentrations were observed for the feed category 'forages and roughage, and products derived thereof', in particular for clover meal and lucerne and for the feed category 'Tubers, roots and products derived thereof', in particular for potatoes. No nitrate occurrence data were reported for 'Compound feed'.

Table 6: Summary statistics of nitrate in feed

Feed category (level 1)	Feed category (level 2)	N	%LCD	Concentration range (LB–UB) (mg NO_3^-/kg)[a]			
				Mean	Median	P75	P95
Cereal grains, their products and by-products	Cereal grains, unspecified	6	33	19–22	19–19	–	–
	Barley	4	25	127–127	–	–	–
	Wheat	289	12	29–29	19–19	36–36	83–83
	Mixed grains	34	15	43–44	31–31	78–78	–
	Maize	211	34	59–61	15–18	53–53	245–245
	Oats	6	50	819–822	10–14	–	–
Miscellaneous	Starch	80	10	22–24	14–15	24–26	55–55
Oil seeds, oil fruits and products derived thereof	Rape seed	57	32	44–45	22–22	45–45	–
	Toasted soya (beans)	4	25	13–13	–	–	–
	Sunflower seed	479	0	95–95	89–89	97–97	125–125
	Linseed	2	0	28–28	–	–	–

Feed category (level 1)	Feed category (level 2)	N	%LCD	Concentration range (LB–UB) (mg NO_3^-/kg)[a]			
				Mean	Median	P75	P95
Legume seeds and products derived thereof	Peas	4	25	5–6	–	–	–
Tubers, roots and products derived thereof	Carrots	76	14	115–116	45–45	134–134	555–555
	Potatoes	50	2	349–349	211–211	270–270	–
	Sweet potato	1	0	105–105	–	–	–
Forages and roughage, and products derived thereof	Forages and roughage, and products derived thereof, unspecified	6	67	10–14	0–8	–	–
	Lucerne	71	14	869–870	268–268	753–753	4,063–4,063
	Clover meal	26	38	1,068–1,069	132–132	2,709–2,709	–
	Forage meal	135	44	292–295	25–25	178–178	1,384–1,384
	Grass, field dried	1	100	0–2	–	–	–

N: number of analytical results; LCD: left-censored data; P75: 75th percentile; P95: 95th percentile; LB: lower bound; UB: upper bound.
(a): The different percentiles were only described when a minimum number of analytical results were available; 60 results for the 95th percentile, 11 results for the 75th percentile and 6 results for the median. Results obtained on occurrence data with fewer analytical results may not be statistically robust (EFSA, 2011b).

Additional data for nitrate in grass silage and maize silage as referred in Section 3.2.1 are reported in Annex III, Table III.6.

Occurrence data of nitrite

Table 7 provides a summary of occurrence data on nitrite across feed categories including the number of results, percentage of left-censored data and statistical descriptors of the results (mean, median, 75th and 95th percentile). More detail on statistical description is reported in Annex III, Tables III.3–III.5.

The occurrence data on nitrite were available for eight FoodEx level 1 feed categories with a majority of analytical results available for 'tubers, roots and products derived thereof', in particular for sugar beet and 'cereal grains, their products and by-products', in particular for wheat and maize, whilst very limited data were available for other grains. For categories with ≥ 5 analytical results, the highest nitrite mean concentrations were observed for the feed category 'tubers, roots and products derived thereof', in particular for sugar beet molasses. Even higher nitrite mean concentrations were measured for 'other plants, algae and products derived thereof', in particular for sugar cane molasses, but this is based only on four analytical results available (of which two were left censored), and therefore should be considered only indicative. For the remaining feed categories, the nitrite mean concentrations were rather low.

Data were provided for 32 samples of compound/complete feed, but there was no information on the species for which these were manufactured. Therefore, the information could not be used to estimating exposure.

Table 7: Summary statistics of nitrite in feed

Feed category (level 1)	Feed category (level 2)	N	%LCD	Concentration range (LB–UB) (mg NO_2^-/kg)[a]			
				Mean	Median	P75	P95
Cereal grains, their products and by-products	Cereal grains, unspecified	4	100	0–6.5	–	–	–
	Barley	1	100	0–1.0	–	–	–
	Wheat	378	68	4.1–5.6	0–5.0	5.0–5.0	27–27
	Mixed grains	48	85	0.3–1.0	0–0.5	0–2.0	–
	Maize	159	91	1.2–4.0	0–5.0	0–5.0	10–10

Feed category (level 1)	Feed category (level 2)	N	%LCD	Concentration range (LB–UB) (mg NO_2^-/kg)[(a)]			
				Mean	Median	P75	P95
Fish, other aquatic animals and products derived thereof	Fish	26	96	0.2–1.3	0–1.0	0–1.1	–
Miscellaneous	Starch	63	67	3.0–6.2	0–5.0	5.0–5.0	10–10
Oil seeds, oil fruits and products derived thereof	Oil seeds, oil fruits and products derived thereof, unspecified	3	100	0–6.5	–	–	–
	Pumpkin and squash seed	1	100	0–6.5	–	–	–
	Rape seed	31	94	0.1–4.9	0–6.5	0–10	–
	Toasted soya (beans)	1	100	0–6.5	–	–	–
	Sunflower seed	89	33	1.1–1.9	0.8–1.4	2.1–2.3	2.6–10
	Linseed	2	100	0–1.0	–	–	–
Tubers, roots and products derived thereof	Sugar beet	578	13	110–111	68–68	166–166	346–346
	Potatoes	140	9	5.1–5.1	2.0–2.1	4.0–4.1	13–13
Forages and roughage, and products derived thereof	Lucerne	1	100	0–0.04	–	–	–
Other plants, algae and products derived thereof	(Sugar) cane molasses	4	50	180–183	–	–	–
Compound feed	Complete feed	32	38	2.5–3.4	1.3–1.7	2.9–5.2	–

N: number of analytical results; LCD: left-censored data; P75: 75th percentile; P95: 95th percentile; LB: lower bound; UB: upper bound.

(a): The different percentiles were only described when a minimum number of analytical results were available; 60 results for the 95th percentile, 11 results for the 75th percentile and 6 results for the median. Results obtained on occurrence data with fewer analytical results may not be statistically robust (EFSA, 2011b).

(b): Out of these, 577 analytical results were on 'Sugar beet molasses'.

3.2.5. Feed processing

Levels of nitrate in feeds associated with nitrite poisoning of farm livestock are normally found in forage crops. These may be fed without any processing, i.e. by grazing, or conserved by ensiling or drying. Forages harvested for ensiling frequently contain appreciable amounts of nitrate, but during fermentation, the nitrate is partially or completely degraded to ammonia and nitrous oxide, with nitrite and nitric oxide occurring as intermediates. The loss of nitrate during ensiling appears to be related to how long the crop remains at a pH at which enterobacteria may grow and utilise nitrate (Driehuis et al., 2018). Thus, silages produced from grasses with high sugar contents resulting in rapid reductions in pH may retain more nitrate than those with delayed fermentation.

Field drying of grass for hay may result in small increases in nitrate-nitrogen concentrations (Singer, 2002).

3.3. Occurrence data on unprocessed food of animal origin

The CONTAM Panel considered that it would be of interest to estimate the presence of nitrate nitrite and N-nitrosamines in foods of animal origin where the presence in not due to the intended use of the additive or as a consequence of a further processing of the raw materials but as a result of a carry-over from feed or from internal nitrosation.

For this purpose, it was explored if analytical results on nitrate and nitrite were reported for unprocessed food of animal origin within the EFSA database. Totally, 252 analytical results (96 for nitrate, 3 for nitrite, 21 for potassium nitrate, 52 for sodium nitrate and 80 for sodium nitrite) were identified. These included mainly samples on meat and several samples on milk but not in eggs.

A majority of the available data in meat were transmitted to EFSA within the call for food additives, and therefore, it is likely that the classification of meat products as unprocessed is not accurate and nitrate and nitrite were intentionally added to the meat food products as food additives during the processing. The remaining data set included limited number of samples on meat (13 and 10 samples for nitrate and nitrite, respectively) with particularly high concentrations (up to for 313 mg/kg NO_3^- and up to 99 mg/kg NO_2^-) implying also the presence of food additives. The Panel considered that it is not possible to ensure that the nitrate and nitrite in the meat originate from feed and not from food processing and therefore could not draw any conclusion.

Very low nitrate and nitrite levels were reported in the milk.

No analytical data in unprocessed food of interest were reported on N-nitrosamines.

3.4. Exposure assessment

3.4.1. Previously reported exposure assessments in animals

In 2009, EFSA reviewed nitrite as an undesirable substance in animal feeds and estimated the exposure by farm animals. Data on levels of nitrite in 94 samples of feed were received from 3 European countries covering the period 2002–2008, but they were considered insufficient to estimate exposure based on intakes of these feeds for non-ruminants. Therefore, exposures were estimated based on the maximum permitted sodium nitrite concentration in feeds, and the maximum limit value for water. Estimated nitrite exposures ranged from 0.26 (sows) to 0.71 (broiler chickens) mg/kg bw per day. For ruminants, the estimated exposure was based on the highest values of nitrite in samples of forage (26.2 mg/kg DM) and compound feed (11.36 mg NO_2^- /kg DM) reported from Slovenia, together with the maximum permitted limit for water. The highest estimated exposure was for lactating dairy cows (0.87 mg/kg bw per day) (EFSA, 2009). It was also noted that a small but significant exposure to nitrite can also occur in grazing animals due to soil ingestion, which has been estimated to vary between 1% and 18% of the feed DM intake (Thornton and Abrahams, 1983).

3.4.2. Dietary exposure assessment for farm and companion animals

3.4.2.1. Nitrate

For many livestock in the EU, and in particular non-ruminant livestock and companion animals, diets consist largely or entirely of manufactured compound feeds. However, no data on levels of nitrate in compound feeds for the specific livestock categories were available, and therefore, exposures have been estimated using example rations and concentrations in individual feed materials (see Annex II for details). According to EFSA (2011b), caution is needed when calculating exposure of the 95th percentile where data on less than 60 samples are available, since the results may not be statistically robust. Since there were insufficient data for the main feed materials used for livestock, exposure estimates have been made for the mean lower bound (LB) and upper bound (UB) concentrations only.

Forages are essential components of the diets of ruminants and horses. Although the EFSA database included nitrate levels for 135 samples in the category 'forage meal (grass meal, green meal)', this was considered too imprecise to use to estimate exposure. No reliable data on nitrate levels in fresh grass have been identified, and therefore, no estimates of exposure have been made for fresh grass-based diets. Data on nitrate levels in grass silage have been obtained from an international group of laboratories testing agricultural commodities; this reported a mean nitrate content in samples analysed in the UK during 2015–2019 of 2,600 mg/kg DM, a value which is similar to that reported for grass silages analysed in the Netherlands between 2014 and 2018.[25] For maize silage, a value of 880 mg NO_3^-/kg DM has been assumed, which is the mean of samples analysed in the Netherlands between 1997 and 2002 (Van den Top, 2005) (see also Section 3.2.1).

Estimates of exposure to nitrate (mean LB and UB) from feed by ruminants, and non-ruminants and companion animals, are given in Tables 8 and 9, respectively.

In addition to feeds, water represents a potential source of nitrate. Although water consumption can vary significantly, a number of authorities have published estimated intakes by livestock. For this Opinion, estimates of daily water consumption are taken from OMAFRA (2007), EFSA (2009) and Defra,[26] and values used are given in Annex III. No occurrence data on nitrate content in water

[25] Source: Eurofins UK Limited, *Personal communication*; Eurofins Netherlands. https://www.melkvee.nl/site/assets/files/0/84/070/graskuilned2018_1007voorjaar.pdf

[26] Department of Food and Rural Affairs (Defra), UK, Project WU0132: 'Sustainable Water for Livestock'.

consumed by animals were identified. In order to estimate nitrate intake from water, the mean LB/UB concentrations of nitrate in tap water of 14.8/14.9 mg/L, respectively, were retrieved from the EFSA database on chemical occurrence data. Estimates of intake of nitrate from feed, water, as well as from feed and water combined, are given in Tables 8 and 9.

Table 8: Estimated exposure of ruminants to nitrate in feed and water

Animal category	Forage	Approach*	Nitrate					
			In feed			In water		In feed and water
			Diet concentration (mg NO_3^-/kg DM)	Intake (mg NO_3^-/day)	Intake (mg NO_3^-/kg bw)**	Intake (mg NO_3^-/day)	Total intake (mg NO_3^-/day)	Total intake (mg NO_3^-/kg bw)**
Dairy cows: high yielding	Grass silage	LB	1,578	32,673	50	1,781	34,454	53
		UB	1,579	32,680	50	1,793	34,454	53
	Maize silage	LB	538	14,683	23	1,781	16,464	25
		UB	539	14,701	23	1,793	16,494	25
Beef: fattening	Grass silage	LB	2,221	21,323	53	445	21,768	54
		UB	2,221	21,324	53	448	21,772	54
	Maize silage	LB	665	4,386	15	448	4,835	16
		UB	665	4,389	15	448	4,837	16
Sheep: lactating	Grass silage	LB	1,323	3,703	47	104	3,807	48
		UB	1,323	3,704	47	105	3,809	48
Goats: lactating	Grass silage	LB	712	2,422	40	223	2,645	44
		UB	713	2,424	40	224	2,648	44
Goats: fattening	Grass silage	LB	1,589	2,383	60	89.0	2,472	62
		UB	1,589	2,384	60	89.6	2,473	62
Horses	Maize silage	LB	643	5,790	13	667	6,458	14
		UB	644	5,795	13	672	6,467	14

*: See Section 2.2.
**: Values rounded to significant digits.

Table 9: Estimated exposure of non-ruminants to nitrate in feed and water

Animal category	Approach*	In feed			In water	In feed and water	
		Diet concentration (mg NO_3^-/kg DM)	Intake (mg/day)	Intake (mg NO_3^-/kg bw)**	Intake (mg NO_3^-/day)	Total intake (mg NO_3^-/day)	Total intake (mg NO_3^-/kg bw)**
Pig: starter	LB	39.6	39.6	2.0	29.7	69.3	3.5
	UB	40.6	40.6	2.0	29.9	70.5	3.5
Pig: finisher	LB	36.4	109	1.0	148	258	2.4
	UB	37.4	112	1.1	149	262	2.5
Sows: lactating	LB	23.5	141	0.7	371	512	2.2
	UB	24.5	147	0.7	374	520	2.2
Chickens: fattening	LB	56.6	6.79	3.4	2.23	9.01	6.9
	UB	57.7	6.93	3.5	2.24	9.17	6.9
Chickens: laying	LB	91.8	11.0	5.5	1.71	12.7	7.9
	UB	92.9	11.1	5.6	1.72	12.9	8.0
Turkeys: fattening	LB	143	57.3	4.8	10.4	67.7	5.8
	UB	144	57.6	4.8	10.5	68.1	5.8

Animal category	Approach*	In feed			In water	In feed and water	
		Diet concentration (mg NO_3^-/kg DM)	Intake (mg/day)	Intake (mg NO_3^-/kg bw)**	Intake (mg NO_3^-/day)	Total intake (mg NO_3^-/ day)	Total intake (mg NO_3^-/kg bw)**
Ducks: fattening	LB	75.2	10.5	3.5	17.8	28.3	9.5
	UB	76.1	10.6	3.5	17.9	28.6	9.5
Cats	LB	17.0	1.02	0.2	2.97	3.99	1.0
	UB	17.5	1.05	0.3	2.99	4.04	1.0
Dogs	LB	24.9	8.96	0.4	20.8	29.7	1.2
	UB	25.5	9.17	0.4	20.9	30.1	1.2
Rabbits	LB	33.2	4.98	2.5	2.97	8.0	10
	UB	33.7	5.06	2.5	2.99	8.0	10

*: See Section 2.2.
**: Values rounded to significant digits.

3.4.2.2. Nitrite

For most of the primary feeds used in diets of farm or companion animals, and particularly cereal grains, oilseed meals and forages, no data on levels of nitrite were available. The CONTAM Panel concluded therefore that it was not appropriate to estimate exposures by farm livestock or companion animals to nitrite.

3.4.3. Conclusions of the exposure assessment

3.4.3.1. Nitrate

Exposure estimates in this Opinion are derived from a limited number of data in the EFSA database for nitrate in commonly used feed materials for farm and companion animals. Cereals are major ingredients in livestock diets, but levels of nitrate in cereal grains were derived from very few samples (< 6 samples each for wheat, barley or oats). As a result, only mean lower bound and upper bound estimates of exposure have been possible. The absence of any data for oilseed meals, which may be included at levels of up to 30% in complete diets of non-ruminants or complementary feed for ruminants, markedly affects the estimates of exposure. No published data on levels of nitrate in cereal grains or oilseed meals were identified with which to augment the EFSA data and improve exposure estimates.

For ruminants and horses, both fresh and conserved forages are essential ingredients of their diets, and frequently account for their only feed. However, the EFSA database contained no useful data on levels of nitrate these feeds. Limited information on levels of nitrate in grass and maize silages were obtained from unpublished sources, and this was used to estimate exposure on grass silage-based diets. No reliable data were identified for fresh grass, and therefore, no estimates of exposure were possible for livestock on grazed grass.

In this context, it should be noted that during silage fermentation nitrate are reduced to nitrite and ammonia. As a result, nitrate in grass silages will be lower than in the grasses from which they were made (Spoelstra, 1987), and therefore, direct extrapolation of exposure from silage-based diets to grazed grass is unreliable. Furthermore, there is considerable between- and within-year variation in nitrate levels in fresh and conserved herbage due to climatic variation and management factors (den Top, 2005; Wilkinson et al., 2014). For example, mean nitrate contents of grass silages analysed in the UK ranged from 2.1 (2019) to 3.7 (2017) g NO_3^-/kg DM, while in the Netherlands for the same period they varied from 1.6 (2015) to 3.9 (2018) g NO_3^-/kg DM.[23]

For maize silages, Van den Top (2005) reported levels ranging from 0.0 to 2.3 g NO_3^-/kg DM for samples analysed between 1997 and 2002.

The level of nitrogen applied to grassland also has a major effect on nitrate levels in forages.

Nitrogen application rates in particular have a major impact on levels of nitrate in forage crops. In response to EU Directives,[27] nitrogen fertiliser use (kg/ha) in the EU declined between 1990 and 2010,

[27] In particular the Nitrates Directive (1991) and the Water Framework Directive (2000).

but in recent years has increased,[28] resulting in likely increases in nitrate levels in herbages. Therefore, extrapolation of levels in fresh herbage from old data could be misleading. Overall, and given the limitations of the database and the wide range of factors that influence the levels of nitrate in feed materials, estimates of total exposure should be interpreted with caution.

Water can be a major source of exposure in farm animals. Published estimates of intake vary considerably due to the many factors that affect water consumption (body weight and metabolic activity, moisture content of the feed and climatic conditions). In this Opinion, water intakes proposed by EFSA (2009) and OMAFR[29] have been used, together with levels of nitrate in tap, to estimate exposure to nitrate in water. However, many animals, particularly grazing ruminants and horses, the source of water may be from on-farm streams, ponds and wells, for which levels of nitrate/nitrite are unknown.

Soil ingestion by grazing animals may account for up to 18% of their daily dry matter intake (Thornton and Abrahams, 1983). Levels of nitrate nitrogen in soils can vary considerably, influenced by many inter-related factors including the soil type, fertiliser application, previous and current crops, the extent of nitrification by nitrifying bacteria and climatic conditions (Brady and Weil, 1996). The estimates presented here do not take account of potential intake of nitrate in soil by grazing animals.

3.4.3.2. Nitrite

There were insufficient data on levels of nitrite in feeds to allow the CONTAM Panel to reliably estimate the exposures of livestock and companion animals.

3.5. Risk characterisation

There is limited knowledge on the effects of nitrate and nitrite in farm and companion animals. Furthermore, there is no comprehensive database on feed consumption by livestock in the EU. The data on levels of nitrite in feeds were insufficient to allow the calculation of exposure estimates and do not allow appropriate risk characterisation. Due to limited data, the chronic exposure to nitrate from animal diets could only be estimated at the mean level, using expected feed intakes and example diets.[30] For rabbits, poultry, horses, dogs, cats, fish and fur animals, the health risk from the exposure to nitrate and nitrite could not be assessed as no NOAELs or LOAELs have been identified. It has, therefore, not been possible to fully assess the risks of nitrate and nitrite for farm and companion animal health.

Risk characterisation of nitrate was performed in ruminants and pigs. The exposures to nitrate have been compared with identified reference points ($BMDL_{10}$ and NOAEL expressed as mg/kg bw per day). The identified reference points for ruminants and pigs were used for risk characterisation. In Table 10, mean exposure estimates are presented together with NOAEL.

Increase in MetHb formation has been selected as the critical effect in cattle. A $BMDL_{10}$ of 64 mg NO_3^-/kg bw per day was identified based on MetHb levels of 10% in the blood of animals, above which clinical signs start. This reference point was considered protective for other effects resulting from nitrate intake except for reproductive effects in pregnant cows.

This $BMDL_{10}$ was compared with estimated mean UB exposures of 50 and 53 mg NO_3^-/kg bw per day in feed (53 and 54 mg NO_3^-/kg bw per day for feed plus water) for dairy cows and beef cattle, respectively, on grass silage-based diets. The same $BMDL_{10}$ was also compared with estimated mean UB exposures of 46 and 60 mg NO_3^-/kg bw per day in feed containing grass silage for lactating sheep and fattening goats, respectively. In goats, the exposures estimates were only marginally lower than the reference point. It was noted, therefore, that nitrate levels in diets of ruminants fed grass silage-based diets may raise a health concern.

As noted above, there can be large between-year differences in nitrate levels in grass silage. In this Opinion, a 5-year mean concentration (2.6 mg NO_3^-/kg DM) has been used to estimate exposure, but using the highest annual mean concentration (3.9 mg NO_3^-/kg DM in the Netherlands in 2018) would have resulted in all grass silage-based diets exceeding the $BMDL_{10}$ of 64 mg NO_3^-/kg bw per day.

For non-ruminants, an NOAEL of 410 mg NO_3^-/kg bw per day was identified for pigs. This NOAEL compares with estimated mean UB exposures of 2.0 mg NO_3^-/kg bw per day from feed for starter pigs. Even when nitrate in water is included, mean UB exposures were 3.5 mg NO_3^-/kg bw per day for starter pigs.

[28] Source: Eurostat 'Agri-environmental indicator – mineral fertiliser consumption'.
[29] OMAFR: Ontario Ministry of Agriculture, Food and Rural Affairs Factsheet 716/400, 2019.
[30] See Annex II for details of calculations.

The Panel concluded that the exposure estimates of nitrate consumption for pigs appear very low compared to the reference point for this species, although the absence of data on certain key ingredients in their diets is likely to have resulted in an underestimation of the levels of exposure.

Table 10: Comparison of nitrate and nitrite exposure levels and reference points for different farm and companion animal species

Species	Critical effect	Reference point mg ion/kg bw per day	Estimated exposure in feed (mg ion/kg bw per day	Estimated exposure, % of reference point
			Mean (UB)	Mean (UB)
NO_3^-				
Ruminants – **Dairy cows**	Increase in MetHb (above 10%)	64 ($BMDL_{10}$)	50/23 (grass silage/maize silage)	78/36 (grass silage/maize silage)
– **Beef cattle**			53/15 (grass silage/maize silage)	83/23 (grass silage/maize silage)
– **Sheep (lactating)**			46 (grass silage)	72(grass silage)
– **Goats (fattening)**			60 (grass silage)	94 (grass silage)
Pigs	Performance and blood parameters	410 (NOAEL)	2.0 (Pig starter)	< 1.0 (Pig starter)
NO_2^-				
Pigs	Clinical signs	20 (NOAEL)	–	–

BMDL: benchmark dose level; MetHb: methaemoglobin; NOAEL: no observed adverse effect level; UB: upper bound.

3.6. Uncertainty analysis

The uncertainty analysis includes a qualitative assessment of whether each source of uncertainty affecting different parts of the risk assessment leads to over/underestimation of the resulting risk.

3.6.1. Uncertainty in occurrence and exposure

Fresh (grazed) forages are the major ingredients in diets of ruminants and horses for much of the year, but due to the absence of data on levels of nitrate and nitrite in fresh herbage, it has not been possible to estimate exposure for these animals.

Data on the occurrence of nitrate in grass silage, obtained as part of industry monitoring programmes, were from only two countries, and therefore may not be representative of silages made in other EU countries. The nitrate data for non-forage feeds were mainly reported by only three countries and nitrite data only by one country, while other countries submitted only limited number of data. There is an overall uncertainty in possible regional differences in nitrate and nitrite contamination of feed commodities and it is evident that the data set is not fully representative for feed in the EU.

Due to the lack of information on recovery rates, some of data were not corrected for recovery which may have led to an underestimation of the present assessment.

Samples with left-censored data introduced uncertainties to the overall exposure estimate since the use of the LB in this assessment tends to underestimate, while UB tends to overestimate the dietary exposure. In addition, several analytical results were reported with relatively high LOQs which may have an impact on the UB estimations when dealing with left-censored data. However, the impact resulted to be minor since the data set comprised only low proportion of left-censored data.

Overall, the animal exposure assessment was hampered by the limited occurrence data on nitrate and nitrite in feeds. For nitrite, no data were available for the major cereals (wheat, barley, oats or maize), for soybean meal (which together with cereals may account for more than 50% of the diet for of non-ruminant animals) or forages which are a major ingredient in ruminant diets. As a result, the CONTAM Panel were unable to estimate exposure to nitrite. For nitrate, there were limited numbers of

samples for the major cereals, while data for forages were only from two European countries, and completely absent for nitrite.

For many livestock, particularly grazing ruminants and horses, water consumption may be from on-farm wells and streams, for which levels of nitrite/nitrate are unknown.

3.6.2. Uncertainty in the studies used for evaluation of the adverse effects in farm and companion animals

For all the animal species taken into consideration, no data were available on the possible differences among breeds.

Based on the current literature, there is uncertainty regarding the successful adaptation of cattle to nitrate in feed. There are insufficient grounds to set a different reference point of nitrate for cattle which have undergone long-term exposure to elevated levels of nitrate; however, several factors (breed, feed regime, ruminal microflora) lead to increased tolerance to nitrate. Therefore, the reference point calculated for non-adapted cattle may lead to overestimation of the risk under current feeding practices.

The effects on milk quality parameters (reduced protein and fat content) can occur when lactating cows are fed high doses of nitrate; however, the available data are limited.

Reproductive adverse effects in pregnant cows due to nitrate consumption were indicated in several publications; however, the underlying mode of action and the exposure levels are not clear.

The $BMDL_{10}$ value of 64 mg NO_3^-/kg bw per day was applied to conservatively estimate the health risk to ovines and caprines, considering that they do not seem to be more sensitive than bovines. However, the degree of sensitivity is uncertain based on the current data.

Limited toxicological data are available on the adverse effects of nitrate in the feed in pigs.

3.6.3. Summary of uncertainties

In Table 11, a summary of the uncertainty evaluation is presented, highlighting the main sources of uncertainty and indicating an estimate of whether the source of uncertainty leads to over/underestimation of the resulting risk.

Table 11: Summary of the qualitative evaluation of the impact of uncertainties on the assessment of nitrate and nitrite

Sources of uncertainty	Direction[(a)]
In hazard identification and characterisation of nitrate and nitrite	
No differences between breeds were considered	+/–
Bovines	
Adaptation methods leading to reduction of acute nitrate exposure	+
Scarce data on dairy cows' sensitivity to nitrate related to milk production	+/–
Nitrate levels inducing reproductive effects in pregnant cows	–
Ovines and caprines	
Degree of sensitivity compared to cattle	+
Pigs	
Limited data to support the calculation of reference points for pigs	+/–
In occurrence data	
Imputation of mean value on grass and maize silage based on reported means from two countries	+/–
Extrapolation of occurrence data from few Member States to whole EU for feeding items included in the exposure calculations	+/–
Use of standard estimates of feed intakes and assumed diet compositions for farmed and companion animals	+/–
Absence of data on fresh grass	–
In exposure assessment of nitrate	
Use of feed ingredients instead of compound feed data due to lack of information of the target animal for compound feed	+/–

Sources of uncertainty	Direction[(a)]
High variability of feedstuffs used and feeding systems for livestock	+/–
Limited consumption data for certain animal species	+/–
Water contribution emphasised due to lack of data on all feed sources	+
Levels of nitrate/nitrite in sources other than tap water are unknown	–

(a): + = uncertainty with potential to cause overestimation of exposure/risk; – = uncertainty with potential to cause underestimation of exposure/risk, +/– = extent of potential over/underestimation might differ in direction.

Overall, the uncertainties in the risk assessment of farm and companion animals are large.

4. Conclusions

Hazard identification and characterisation

Toxicokinetics

Ruminants

- Via a metabolic pathway involving bacterial NADH- or FADH-nitroreductases, nitrate is reduced first to nitrite and then mainly to ammonia, which represents an important nitrogen source for bacterial protein synthesis.
- A gradual increase of nitrate in ruminant diets has been reported to induce adaptive changes in the gut microbiome, leading to an increased conversion of nitrate to nitrite and then to ammonia. However, in many cases, the increase in the rate of the former reaction seems to prevail over the conversion of nitrite to ammonia, resulting in only limited reduction in blood MetHb in the animal.
- There is a rapid and dose-related absorption of nitrate and nitrite, with a complex interconversion between the two anions followed by a rapid excretion mainly via the urine.
- The transfer of nitrite to fetal blood has been demonstrated in cows.
- The transfer of nitrate and nitrite from feed to animal products was found to be negligible from the scarce data available.

Non-ruminants

- In pigs, the extent of nitrate reduction to nitrite is much lower than in ruminants. Besides the intestine, it takes place also in the oral cavity due to an extensive salivary recirculation of nitrate.
- In horses, little is known about the kinetics of nitrate/nitrite. Nitrate reduction to nitrite is brought about by an active caecal and colonic microflora and is reported as intermediate between ruminants and pigs.
- No relevant data on the kinetics of dietary nitrate/nitrite in rabbits, poultry, dogs, cats, fur animals and fish have been identified in the literature.

Mode of action

- Nitrate is less toxic than nitrite.
- Nitrite is able to oxidise haemoglobin to methaemoglobin, which has a very limited oxygen carrying capacity thereby causing tissue hypoxia.
- Nitrate acts as a competitive inhibitor of the sodium-iodide symporter (NIS), leading to a decrease in both iodide uptake and its availability for thyroid hormone synthesis.
- Nitric oxide, generated by nitrite reduction, is thought to be responsible for vasodilation, resulting in blood pressure fall.
- Oxidative stress is mainly linked to the nitrite-mediated generation of peroxynitrite radical and other free radicals.
- The MoAs underlying other effects (vitamin A and E depletion, abortion, effects on fertility) are still to be unraveled.

Adverse effects

Ruminants

- The generation of MetHb from the reaction between nitrite and oxyhaemoglobin is well established and is considered the mediator of most adverse effects following exposure to nitrate and nitrite in ruminants. The resulting depression of aerobic metabolism can be quantitatively associated with many clinical signs, such as cyanosis, hypoxaemia, tachycardia, dyspnoea, incoordination, muscle tremors and death.
- The association of MetHb formation with reproductive effects in pregnant cows such as late abortions and still births has not been clearly demonstrated.
- A $BMDL_{10}$ (64 mg nitrate/kg bw per day) was identified in adult cattle based on a BMR of 10% of MetHb related to total Hb in the blood, which the Panel considered as the threshold for emergence of clinical signs.
- The $BMDL_{10}$ defined for adult cattle is also applicable for lactating cows and calves.
- Ovines and caprines have not been demonstrated to be more sensitive to nitrate than bovines. Therefore, the $BMDL_{10}$ identified for adult cattle may also be applied for these animal species.
- The available data do not permit the establishment of reference points for nitrite in ruminant species.

Non-ruminants

- An NOAEL of 410 mg nitrate/kg bw per day for nitrate was identified at the highest dose tested for effects on animal performance and haematological parameters in pigs. An NOAEL of 20 mg nitrite/kg bw per day was also identified based on the absence of clinical signs.
- No reference points for nitrate and nitrite were identified for rabbits, poultry, horses, dogs, cats and fish.

Occurrence and exposure

- The final data set contained 1,542 nitrate analytical results and 1,561 nitrite analytical results obtained between 2010 and 2019 and sampled in 15 different European countries. A part of the analytical results referred to the EU countries or EEA-countries without specification of the country.
- The proportion of LCD observed for nitrate was 16%, whilst the data set of nitrite comprised 43% LCD data.
- The highest mean nitrate concentrations were observed for the feed category 'forages and roughage, and products derived thereof', in particular for clover meal and lucerne and for the feed 'Tubers, roots and products derived thereof', in particular for potatoes. For categories with $\geq$ 5 analytical results, the highest mean nitrite concentrations were observed for the feed category 'tubers, roots and products derived thereof', in particular for sugar beet molasses.
- Estimates of exposure to nitrate are hampered by the lack of data for many of the feeds and feed materials commonly used in the diets of farmed and companion animals. Therefore, all estimates are likely to be underestimates.
- Due to insufficient data on levels of nitrite in feeds, no reliable estimates of exposure could be calculated.

Ruminants

- Nitrate toxicity is most commonly reported in ruminants fed fresh herbage; due to the absence of any data on nitrate levels in fresh grass, it has not been possible to estimate exposure for those livestock most susceptible to nitrate toxicity.
- The highest estimated dietary exposure of cattle to nitrate from feed was for beef cattle fed a grass silage-based diet (53 mg nitrate/kg bw per day).
- For sheep and goats, the highest estimated dietary exposure to nitrate from grass silage-based diet was 46 and 60 mg nitrate/kg bw per day, for lactating sheep and fattening goats, respectively.

Non-ruminants

- The Panel concluded that the exposure estimates to nitrate for non-ruminants appear low (from mean UB 0.3 mg nitrate/kg bw per day in cats to 5.6 mg nitrate/kg bw per day in laying

chicken). However, this is probably due to the lack of data on the main ingredients in their diets.

Formation of N-nitrosamines

- A limited number of old studies in few animal species showed a very low *in vivo* formation of N-nitrosamines if animals were administered high amounts of both nitrite and secondary amines, i.e. under feeding conditions unlikely to be met under normal feeding regimes.
- The transfer to milk of N-nitrosamines, either endogenously formed or already present in feed, seems to be negligible, based on few experiments.
- The dietary supplementation of beef cattle with nitrate at dosages effective in mitigating methane emissions did not result in the accumulation of measurable amounts of N-nitrosamines in meat.
- The CONTAM Panel considered that although N-nitrosamines could be produced endogenously, the evidence to assess the risk is very limited and that there is no information to link this endogenous production of N-nitrosamines with adverse effects in farmed and companion animals.

Risk characterisation

- The risk characterisation of exposure to nitrate and nitrite was evaluated taking into consideration the comparison between the mean UB exposure estimates and the identified reference points for adverse effects.
- The health risk from the exposure to nitrite in farmed and companion animals could not be assessed as no reference points and/or exposure estimates were available.
- The transfer of nitrate, nitrite and N-nitrosamines produced due to nitrite in feed is likely to be negligible based on the scarce data available.

Ruminants

- A $BMDL_{10}$ of 64 mg nitrate/kg bw per day in adult cattle was identified based on MetHb levels in the blood of animals that would not induce clinical signs of hypoxia. This reference point was considered protective for other effects caused by nitrate intake. The $BMDL_{10}$ is compared with highest estimated mean exposure of 53 mg nitrate/kg bw per day for grass silage for beef cattle. This comparison indicates that the exposure may raise a health concern, considering the uncertainty in the high exposure estimate for grass silage and for other forages that may contain relatively high levels of nitrate but for which data are missing.
- There was insufficient information to set reference point for nitrate for ovines and caprines. However, the data available suggest that they are not more sensitive than bovines. Therefore, the CONTAM Panel considers that the $BMDL_{10}$ value of 64 mg nitrate/kg bw per day could also apply to conservatively estimate the health risk to ovines and caprines. The highest estimated mean exposure to nitrate was found to be of 60 mg nitrate/kg bw per day from grass silage in fattening goats, which may raise a health concern since other diets (including fresh grass) for which data are missing may contain higher levels of nitrate.
- There are some examples in the literature indicating successful adaptation of the ruminants to nitrate in feed, suggesting that the $BMDL_{10}$ calculated may be conservative. However, due to the large variability in the design and outcome of these studies, it is not possible to set a different reference point for animals which have undergone long-term exposure to elevated levels of nitrate.

Non-ruminants

- NOAEL of 410 mg nitrate/kg bw per day was identified for pigs. This compares with estimated mean exposure of 2.0 mg nitrate/kg bw per day from feed for pigs for fattening, which represents less than 1% of the NOAEL values, and therefore, no health concern was raised.
- Reference points for nitrate and nitrite have not been identified for rabbits, poultry, horses, dogs, cats and fish, and therefore, a risk characterisation could not be performed.

5. Recommendations

More information is needed on nitrate and nitrite regarding:

- toxicokinetics in animal species other than ruminants and pigs.

- their adverse effects in animal species other than ruminants and pigs at realistic exposure levels.
- occurrence of nitrate and in particular of nitrite and N-nitrosamines formed due to the presence of nitrate and nitrite in the different major feeds, especially in forages, in order to produce reliable exposure estimates.
- occurrence in feeds for rabbits, horses, poultry, dogs, cats, fur animals and fish.
- occurrence in fresh and ensiled herbages; these feeds are analysed annually by EU commercial laboratories for livestock farmers, and access to these data should be sought in order to better estimate exposure by ruminant livestock and horses.
- endogenous formation of N-nitrosamines in the different species and their transfer to food products of animal origin.
- transfer of nitrate, nitrite and N-nitrosamines formed due to the presence of nitrate and nitrite in feed to food products of animal origin.

6. Documentation as provided to EFSA

1) Nitrite research project: Final report. August 2019. Submitted by Südzucker AG.
2) Benu I, Callaghan MJ, Tomkins N, Hepworth G, Fitzpatrick LA and Parker AJ, 2016. Raw data provided by A. Parker in November 2019 and they have been used for the BMDL calculation in section 3.1.3.1.

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Abbreviations

AIC	Akaike information criterion
ALP	Alkaline phosphatase

ALT	Alanine aminotransferase
AOAC	Association of Official Analytical Chemists
AST	aspartate transaminase
BMD	Benchmark dose
$BMDL_{10}$	Benchmark dose lower confidence limit
BMR	Benchmark response
bw	Body weight
CEN	European Committee for Standardisation
CONTAM	Panel on Contaminants in the Food Chain
DATA Unit	EFSA former EFSA Dietary and Chemical Monitoring Unit
DM	Dry matter
dw	dry weight
EEC	European Economic Community
FADH	Flavin adenine dinucleotide phosphate
fw	fresh weight
GC	gas chromatography
GI	Gastrointestinal
GAP	Good Agricultural Practice
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LB	Lower bound
LD_{50}	Lethal dose killing 50% of the animals
LOAEL	Lowest-observed-adverse-effect-level
LOD	Limit of detection
LOQ	Limit of quantification
MetHb	Methaemoglobin
ML	Maximum level
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NDMA	nitrosodimethylamine
NIS	sodium-iodide symporter
NO	Nitric oxide
NO_3^-	Nitrate ion
NO_2^-	Nitrite ion
NOAEL	no-observed-adverse-effect-level
OECD	Organisation for Economic Co-Operation and Development
PCV	Packed cell volume
SD	Standard deviation
SCF	Scientific Committee on Food
TAC	total antioxidant capacity
TK	Toxicokinetics
UB	Upper bound
WHO	World Health Organization
WI	Water intake

Appendix A – Summaries of reviewed studies

Table A.1: Reported strategies to maintain asymptomatic methaemoglobin concentration in bovines, ovines and caprines

Authors	Species	Feeding method	Treatment dose (mg NO_3/kg bw)	Methaemoglobin (%)	Comments
Fractionation of nitrate in diet					
Benu et al. (2016)	Bovine	NO_3 bolus Average bw = 320 kg	94	11^P	*Bos Indicus* steers given 30, 40 or 50 g bolus of NO_3 once a day or split into two equal portions throughout the day on a basal diet of *Iseilema* sp. hay. Less MetHb when dose is divided into 2 equal portions
			125	$21–53^P$	
			156	$29–60^P$	
Guyader et al. (2015)	Bovine	NO_3 administered in concentrate portion of Hay 50%/50% Concentrate diet. bw 656 kg, DMI 12.4 kg per day	141	26^P	Non-lactating Holstein cows fed linseed and 3% calcium nitrate in concentrate ($5Ca(NO3)2{\cdot}NH_4NO_3{\cdot}10H_2O$; 75% NO_3^- in DM). Delivery of concentrates containing NO_3 was fractionated into 5 portions/day. A total of 60% concentrate from 08.00 to 09.30 and 40% concentrate from 1,600 to 1,630. MetHb measured 3 h after morning dose (60%)
Lee et al. (2015b)	Bovine	1.09% NO_3 treatment given in total mixed ration	130–170	3.35^P	Beef heifers sampled at 3 h and 6 h after feeding. Restricted feeding caused 63% of total diet consumption in first 3 h after feeding compared with 25% of total diet consumed with ad libitum feeding. Greater MetHb in restricted fed heifers
Nolan et al. (2010)	Ovine	4% added KNO_3 was sprinkled as a solution onto oaten hay while the hay was tossed in a rotary feed mixer. The daily ration (1 kg/day air-dry feed) delivered in equal portions each hour by automatic feeders	650	0.62	The sheep were gradually acclimated to the nitrate-containing diet over 18 days. Time of blood sample was not stated. MetHb did not exceed 2.8% and was not different compared with the control sheep
Encapsulation of Nitrate					
Lee et al. (2015a)	Bovine	Treatments from encapsulated nitrate at 0.1–4.8% administered in a total mixed ration	230–917	$2–17^P$	Beef heifers given encapsulated nitrate (EN). With intake restricted (Exp. 1), EN at 4.8% and 5.9% in dietary DM decreased DMI and feed consumption rate in which one animal refused the 2% diet. EN 4.8% and 5.8% caused severe sorting. A decrease in ad libitum DMI of 3% EN. One heifer 59% MetHb when 2.9% EN in restricted diet. No comparison with other nitrate sources

Authors	Species	Feeding method	Treatment dose (mg NO_3/kg bw)	Methaemoglobin (%)	Comments
Silveira et al. (2019)	Caprine	Treatments of 12.5 and 25 g/kg encapsulated nitrate fed in a total mixed ration containing corn and corn silage	663	0.77[P]	Encapsulated nitrate fed to 21 kg Saanen goats in a TMR delivered in two portions each day. Treatments were gradually introduced over 9 days by doubling the dose every 4 days. Blood samples were drawn at 3 h after the morning feeding
de Raphelis-Soissan et al., 2017	Ovine	Treatments of uncoated NO_3 or paraffin oil or palm oil-coated nitrate pills given as a bolus into the rumen	104	3–6[P]	Sheep given a bolus of 5 g NO_3 into the rumen from one of three sources of nitrate. Paraffin-coated NO_3 caused a significant reduction of MetHb, showing that Paraffin oil is more effective than palm oil in encapsulating NO_3 for the protection of sheep from MetHb
El-Zaiat et al. (2014)	Ovine	Nitrate included in a total mixed ration and fed to the lambs	994	1.10[M]	Concentration of MetHb in nitrate-fed lambs remained < 1.10% of total Hb. Blood was collected at 6 h after feeding. No Treatment, Day or Treatment x Day effects detected for MetHb
Even distribution of nitrate in the diet					
Li et al. (2013)	Ovine	ad libitum feeding of a pellet offered twice daily in two equal portions at 10:00 and 16:30 h	398	0.76[M]	Blood samples taken at 6 h after morning feeding for MetHb concentration. Nitrate diet was introduced over 7 days and fed at 1.9% NO_3 DM
Nguyen et al. (2016)	Ovine	Sprinkling a solution of calcium nitrate onto oaten chaff while the chaff was tossed in a rotary feed mixer	435	5.48[P]	Significant defaunation × NO_3 treatment interaction, indicating that lambs given nitrate and were defaunated had greater concentrations of MetHb. Two lambs had 18.1% and 18.3% MetHb on Day 50. One lamb observed with MetHb of 19.1% on Day 85
van Zijderveld et al. (2010)	Ovine	A Basal diet and concentrate fed daily and hand mixed before feeding	665	< 2.0[P]	Lambs given their ration at 0800 h each day. Gradually introduced NO_3 into diet. At 100% nitrate 2 sheep had MetHb 7 and 3% of Hb. Blood sampled at 3 and 5 h after feeding
Gradually increasing nitrate in the diet					
Duthie et al. (2016)	Bovine	Treatment of 18 g NO_3/kg DM given in a total mixed ration or as a concentrate	470	15[P]	Beef steers given increments of 25% $CaNO_3$/week up to 100% (18 g/kg DM) on day –7. At 25–75% MetHb was similar but 100% caused greater MetHb than 25, 50 or 75%. No comparison with no incremental increase in NO_3
Newbold et al. (2014)	Bovine	Total mixed ration	590	> 20[P]	Four days of control feed then NO_3 increased every 4 days up to 3.00% d 21. Nine animals removed for exceeding > 20% MetHb. No comparison with no gradual increase in NO_3

Authors	Species	Feeding method	Treatment dose (mg NO_3/kg bw)	Methaemoglobin (%)	Comments
Tomkins et al. (2018)	Bovine	Flinders grass (*Iseilema* spp.) hay once a day. 6 days only hay, $CaNO_3$ was mixed with a molasses a carrier to encourage intake and presented to each animal before the morning ration	115	15[P]	Supplements were gradually introduced between 7 and 10 days, followed by full dose between 11 and 28 days. No comparison with no gradual increase in NO_3
Van Zijderveld et al. (2011)	Bovine	21 g NO_3/kg DM fed in a total mixed ration. bw = aver. 120.25 kg = approx. 593 kg bw DMI = aver. 19.15 kg DM/day	680	19[P]	Treatments increased each week for 4 weeks in 25% increments. No effect of time or treatment x time interaction. No comparison with no gradual increase in NO_3
Olijhoek et al. (2016)	Bovine	Total mixed ration fed twice daily		1.3–1.6[M]	On day 1–6, the level of dietary nitrate was gradually introduced (incremental increase of 3.5 g of NO_3^-/kg of DM per day) until the planned level was reached. No comparison with no gradual increase in NO_3. Body weight was not reported
Velazco et al. (2014)	Bovine	Total mixed ration	297	3.3[M]	Urea and calcium nitrate progressively increased over 4 weeks from 0.25% and 1% in the starter ration to 0.89% and 2.57% in the finisher ration. No comparison with no gradual increase in NO_3
Godwin et al. (2015)	Bovine	Steers consumed a total mixed ration	655	2[M]	Angus steers fed 1%, 1.5, 2.0, 2.57% of DMI as calcium ammonium nitrate each week up to for 25 days in a TMR. The MetHb concentration increased at day 23 to day 54 to 2% MetHb. It is unknown when the blood sample was taken for MetHb determination in relation to feeding. No comparison with no gradual increase in NO_3
Goopy and Hegarty (2019)	Bovine	ad libitum basal diet of oaten chaff plus a liquid, molasses-based, supplement 29 g N/kg of concentrate. 0.4 kg concentrate consumption/day	213	2.1[M]	7-day adaption period during which the supplement was introduced in two steps from 9 g N/kg on Day 1 to 19 g N/kg on Day 3 and 29 g N/kg on Day 5 and thereafter. No comparison with a g no gradual increase in NO_3. Cattle in NO_3 group had a reduced voluntary intake of the molasses+NO_3 supplement
de Raphélis-Soissan et al. (2014)	Ovine	A solution of nitrate was sprinkled onto oaten chaff in a rotary mixer. NO_3 enriched Oaten chaff was then fed to sheep	620	14[M]	Adapted to nitrate diets from Day 1 to 14, the dose of dietary nitrate being increased every 2 days. Restricted feeding. Sheep initially fed a max of 3%, however, the second day of the experimental period (Day 16), one sheep died unexpectedly from methaemoglobinaemia. Nitrate inclusion levels were reduced from 3.0% to 2.0% in the experiment. No comparison with no gradual increase in NO_3

Authors	Species	Feeding method	Treatment dose (mg NO_3/kg bw)	Methaemoglobin (%)	Comments
El-Zaiat et al. (2014)	Ovine	Total mixed ration was fed to the lambs in two portions per day. Nitrate was included in the TMR	994	1.10^{M}	Sheep fed nitrate were stepwise adapted by replacing 33% of CTL concentrate with nitrate-containing concentrates weekly After 21 day, animals were receiving their final experimental diets. No comparison with no gradual increase in NO_3

P: Peak methaemoglobin concentration defined as blood sampled between 2 and 3 h of feeding NO_3.
M: Mean methaemoglobin concentration defined as all other samples that do not align with peak methaemoglobin concentration, usually sampled before feeding NO_3.

Table A.2: Studies reporting effects in non-ruminants

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Pigs						
Yorkshire pigs, both genders 1st Experiment 5 groups, 8 pigs per treatment Initial bw: 24.7 kg Duration: 84 days 2nd Experiment 5 groups, 8 pigs per treatment Initial bw: 27.6 kg Duration: 84 days	0, 0.75, 1.50 and 3.0 g KNO_3/L drinking water (0, 460, 920, and 1,840 mg NO_3^-)/L drinking water) Water intake group 4 = 3.82 kg per day FI × 3 = 11.46 L Average bw for group 4 (3.0 g KNO_3/L): 51.16 kg Dose for group 4: (410 mg -NO_3 ion)/kg bw/day	In all treatments, no differences in animal performance (bw gain, and feed to gain) and blood parameters (haemoglobin, MetHb and haematocrit)	No pathology was investigated	NOAEL: 3.0 g KNO_3 (~410 mg NO_3 ion)/ kg bw per day	Drinking water	Wood et al. (1967)
	0, 0.2, 0.4, and 0.8 g KNO_2)/L drinking water (0, 108, 216 and 432 mg NO_2/L drinking water) Average bw for group 2 (0.2 KNO_2)/L): 50.70 kg Water intake group 4: FI = 3.53 kg per day FI × 3 = 10.59 L per day. The value of 108 mg NO_2 /L water corresponds to 22 mg NO_2 ion/kg bw per day	In all treatments, no differences in feed gain, haemoglobin, and haematocrit levels. bw gain decreased with the lowest and highest nitrite levels (108, and 432 mg -NO_2 ion/L) against the control, MetHb levels increased with the highest nitrite level (432 mg -NO_2 ion/L) against the control by 5.3-fold	No pathology was investigated	LOAEL: 22 mg NO_2 ion/kg bw per day		

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Yorkshire and Duroc pigs 4 groups, 5 pigs per treatment Initial bw: 32.6 kg Duration: 69 days	0, 25, 50 and 100 mg NO_2-N/L drinking water (0, 82.1, 164.2 and 328.5 mg -NO_2 ion/L drinking water) Average bw for group 2, 3, 4: 59.16, 61.58, 61.58 kg, respectively WI = 4.51, 4.86, 4,73 L per day Approximately 0, 6.25, 12.95 and 25.23 mg NO_2^-/kg bw per day	No effects on bw gain, feed to gain, water intake or liver vitamin A. MetHb levels increased with 50 and 100 mg NO_2-N (164.2 and 328.5 mg -NO_2^-)/L) by 3.77–17 fold compared to 0 and 25 mg NO_2-N (0 and 82.1 mg NO_2^-)/L	No pathology was investigated		Drinking water	Seerley et al. (1965)
Unspecified breed 8- to 10-week-old pigs Acute toxicosis: 21 pigs (treatment groups 1–5 pigs Chronic toxicosis: 10 pigs in control and 6 pigs/ treatment group Duration: 124 days	0.3, 3.0, 6.1, 12.2, 18.3, 19.8, 21.3, 22.9, 24.4, 30.5 mg N- NO_2^-/kg bw per day corresponding to 1, 10, 20, 40, 60, 65, 70, 75, 80, 100 mg NO_2^-/kg bw per day	None (MetHb < 5%) up to 20 mg NO_2^-/kg bw per day, mild (MetHb↑20%) (restlessness, frequent urination, vomiting and detectable dyspnoea) to moderate (MetHb↑48%) (preceded by mild signs and consisted of more pronounced dyspnoea and detectable, cyanosis within 90 min) up to 65 mg NO_2^-/kg bw per day and mortality (MetHb↑75%) from 70 mg NO_2^-/kg bw per day (preceded by moderate signs and consisted of marked dyspnoea and cyanosis followed by coma and death)	No significant gross or microscopic lesions on necropsy, except for a dark brown discoloration of blood, indicating methemoglobinemia	NOAEL: 20 mg NO_2^-/ kg bw per day	Single oral doses (via stomach tube)	London et al. (1967)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
	0, 3, 6.1, 9.1 12.2, 18.3 N-NO_2^-/kg bw per day corresponding to 0, 10, 20, 30, 40, 60 mg NO_2^- ion/kg bw per day	At the highest dose 60 mg NO_2^-/kg bw per day MetHb was between 1.3% and 33.2%, at 40 mg NO_2/kg bw per day between 1.1% and 12.3% and in the other groups below 7.3% at 124 days. The pigs given the highest amount of nitrite frequently developed dyspnoea and cyanosis, with these signs lasting for 1–2 h, after drinking the NO_2 containing water. Inconsistent ↓vitamin A and vitamin E in all nitrate groups ↑total leucocyte count in all pigs given nitrite No effect on average daily gain or feed conversion	No pathological findings		Drinking water	
No study design (Book Chapter)	10–20 mg NO_2^-/kg bw	Cyanosis, high mortality with nitrite levels above 20 mg NO_2^-/kg bw	Blood and muscles with dark brown colour due to MetHb formation		Feed or drinking water	Muirhead et al. (1997)
No study design (Review article)	Oral single doses more than 70 mg NO_2^-/kg bw Oral single doses 40–65 mg NO_2^-/kg bw	Lethal to pigs Not lethal to pigs, but cause clinical symptoms (restlessness, polyuria, vomiting, diarrhoea, dyspnoea, cyanosis, severe breathing difficulties, paralysis, unconsciousness) MetHb increase	Chocolate-brown colour of the blood		Feed and drinking water	Wendt (1985)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
No study design (reference is a veterinary magazine)	Accidental dose 2,000 mg nitrite/L water (600 mg nitrite/kg stomach content) For a default pig bw value of 100 kg (EFSA, 2019) and assuming feed:water ratio from 1:2 to 1:3 the expected dose corresponds to 55–72 mg nitrite/kg bw per day	Lethal to growing pigs (4–6.5 months old) MetHb increase	Chocolate colour of blood		Drinking water	Beilage et al. (2002)
Case 1 140 sows Duration: Few hours Case 2 185 growing pigs bw: 22–25 kg Duration: Few hours Case 3 159 growing pigs bw: ~ 22 kg Duration: 1.5 h	In the troughs, nitrite levels ranged between 1,610 mg/L and 2,430 mg/L water Reported calculation 21 mg nitrite/kg bw	Several sows died, mostly without clinical symptoms. Conjunctivae and the mucosa of the vulva of all the dead sows, and of some of the others, were cyanotic. Nitrite levels in the stomach of the sows were 570 and 1,810 mg/L	Post-mortem examination of two of the sows 2 days after mortality, revealed no significant macroscopic lesions		Water distribution system, pipes	Vyt et al. (2005)
	Reported calculation 70 mg nitrite/kg bw	20 (11%) of them died, mostly without symptoms, sometimes with nervous signs such as paralysis, muscle weakness and lateral recumbency. Brown discoloration of the nose and cyanosis of the conjunctivae were noted. The nitrite levels in the stomachs of 3 pig lets were 49, 164 and 1,420 mg/L	On post-mortem examination, brown discoloration of the blood, indicating severe methaemoglobinaemia, was the single, striking feature		Water distribution system, pipes	
	Water taken at a nipple during the mortality episode contained 570 mg/L nitrite and 270 mg/L nitrate. Bacteriological contamination ($1.88x\ 10^5$ bacteria per mL at 37°C; > 1,000 faecal streptococci /100 mL)	The nitrite level in the stomach of one piglet was 800 mg/L, while the nitrate level was 700 mg/L Several dead piglets	The brown colour of the organs and the blood was prominent		Water that had stayed in the pipes and the barrel for more than 2 months	

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
9 Large White pigs bw: 26.4-36.8 kg Duration: 1 h	Single oral lethal dose 1.385 g sodium nitrite/kg bw) (0.923 g NO_{2-} ion/kg bw) of paste bait (8 pigs consumed 2,170 g paste bait in total, paste bait included 25 g $NaNO_2$ (16.66 g NO_2-)/250 g feed)	The average time to clinical signs first appearing was 17.38 min, and the average duration of symptoms was 42.13 min and included pale extremities, lethargy, ataxia and death	No pathology was investigated		Toxic paste bait	Shapiro et al. (2016)
Rabbits						
162 New Zealand White rabbits: $NaNO_3$: 46 KNO_3: 33 $NaNO_2$: 24 KNO_2: 19 KNO_3 (pregnant): 40 bw: 1.1–2.9 kg Duration: single dose	20% solution of KNO_3 or $NaNO_3$ or a 3% solution of KNO_2 or $NaNO_2$ corresponding to: $NaNO_3$: 1,094 to 2,473 mg NO_3/kg bw KNO_3: 614 to 1,535 mg NO_3/kg bw $NaNO_2$: 100 to 140 mg NO_2/kg bw KNO_2: 51 to 140 mg NO_2/kg bw KNO_3 (pregnant): 737 to 1,105 mg NO_3/kg	Of the 40 pregnant rabbits receiving a single dose of KNO_3 24 died. 13 of the surviving rabbits aborted. The relationship of mortality to dose level was apparent in that all rabbits that received 922 or more mg. NO_3/kg died, and about 50% of those receiving lesser amounts died. Oral LD_{50} for rabbits of $NaNO_3$, KNO_3, $NaNO_2$ and KNO2 was determined to be 1,955, 1,166, 124 and 108 mg NO3- /kg bw Oral LD_{50} for rabbits of $NaNO_2$ and KNO_2 was determined to be 124 and 108 mg NO_2^-/kg bw	No pathology was investigated		Aqueous solution of nitrate and nitrite was administered through a stomach tube to rabbits	Dollahite and Rowe (1974)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
42 New Zealand White male rabbits, 7 rabbits per treatment Duration: 45 weeks (16 to 61 weeks of age) Average initial bw: 2,180 +/- 50.8 g	0, 350 and 700 mg $NaNO_3$ (0, 255 and 510 mg NO_3^-)/L water bw intake and WI as reported in Attia et al., 2018) Approximately 0, 30 and 70 mg NO_3^-/kg bw per day	Rabbits given 510 mg NO_3/L water had significantly lower plasma globulin, red blood cells (RBCs), haemoglobin (Hb), packed cell volume % (PCV%) and total antioxidant capacity (TAC) Testosterone in the blood plasma and the seminal plasma was significantly lower in rabbits given 510 mg NO_3/L water. Digestive, liver and kidney functions were negatively affected in rabbits fed 510 mg NO_3/L water.	No pathology was investigated	NOAEL: 255 mg NO_3/L water or 41.7 mg NO_3^-/kg bw per day.	Drinking water	Attia et al. (2013, 2018)
10 rabbits, 2 per treatment 3.5 to 4 months of age bw: 1.310 kg to 1.720 kg Duration: 120 days	Group A (control): 45 mg $NaNO_3$ (32.8 mg NO_3^-)/L of water, corresponding to 7.35 mg NO_3^-/kg bw per day Group B to E: 100, 200, 400 and 500 mg $NaNO_3$ (72.9, 145.8, 291.7 and 364.7 mg NO_3^-)/L of water, corresponding to 16.40, 32.80, 65.64 and 82.05 mg NO_3^-/kg bw per day All values calculated with default values for CONTAM (EFSA, 2019) and calculating WI = 3 × FI.	Animals were found to be lethargic on 75th day. Rabbits of all groups i.e. A to E showed a continuous increase in heart and respiration rate.	Changes appeared in Group B in the form of mild necrosis of hepatocytes and mild infiltration of inflammatory cells in between the hepatocytes. In higher groups, the liver showed bridging necrosis and portal triditis. Dilatations of central vein with eosinophilic degeneration were observed in Group E only. Mononuclear infiltration in the oesophagus which started in group B and in stomach, histopathological changes appeared in submucosa, muscularis mucosa, muscularis externa and seosa started from group C. D & E: congestion of blood vessels in submucosa and mild infiltration of lymphocytes in muscularis externa.	LOAEL: 16.40 mg - NO_3 ion/kg bw per day	Drinking water	Sharma et al. (2013a,b)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
45 female New Zealand White rabbits (9 rabbits/ treatment) Initial body weight: 900–1,000 g Duration: 14 weeks	Levels of 0, 64, 78.2, 144 and 200 mg nitrate (unspecified)/L drinking water, if ion corresponding to 1.8, 17, 15, 26 and 35 mg nitrate (unspecified)/kg bw per day with default values for CONTAM (EFSA, 2019) and calculating WI = 3 × FI.	In all treatments receiving nitrate, animal performance (body weight, feed intake and water intake) and T3 and T4 blood levels, were adversely affected.	No pathology was investigated		Drinking water	Akasha et al. (2015)
42 male New Zealand White rabbits (6 rabbits/ treatment) Initial body weight: 1,500 g Duration: 40 days	Levels to 0 and 400 mg $NaNO_3$/bw per day (291.76 mg NO_3^-)/kg bw per day, without or with various aqueous garlic extract doses.	The nitrate supplementation increased blood nitrite levels within 10 days after the experiment started until the end of the experimental period, and adversely affected the blood parameters (increase of ALT, AST, ALP, uric acid, urea, creatinine, blood glucose, serum cholesterol and decrease of albumin, total proteins, bilirubin). Aqueous garlic extract supplementation resulted in the reduction of such effects.	No pathological findings. No gross lesions were observed to any treatment group in various organs (liver, kidneys, lungs, brain, heart, intestine, pancreas).		Feed	Rashid et al. (2019)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Poultry						
Cockerels, 40 chickens/ treatment (20 chickens/ replicate pen, 2 replicate pens/treatment) Duration: 28 days	Potassium nitrite at levels equivalent to 0, and 2,165 mg -NO_2 ion/kg feed, without or with various vit. A doses The value of 2,165 mg -NO_2 ion/kg feed corresponds to 130 mg -NO_2 ion/kg bw per day All values calculated with default values for CONTAM (EFSA, 2019)	When comparing the two treatments that were not supplemented with vit. A, the performance of cockerels was adversely affected by the nitrite supplementation (Exp. 1: total bw gain 194 vs. 116 g, feed to gain ratio 2.10 vs. 2.76, increase of the thyroid relative weight 8.8 vs. 15.7 mg/100 g bw; Exp. 2: total bw gain 72 vs. 51 g, increase of the thyroid relative weight 7.7 vs. 17.0 mg/ 100 g bw, higher death rate (Exp. 1: 10 vs. 44%; Exp. 2: 7 vs. 33%))	Enlargement of thyroid gland		Feed	Sell and Roberts (1963)
Turkey poults 4 groups, 50 1-day-old birds per treatment, allocated equally to two replicate pens Initial bw: 40 g Duration: 49 days	0, 3,325, 3,990, and 4,655 mg $NaNO_3$/L drinking water (0, 2,425, 2,910 and 3,395 mg -NO_3 /L drinking water) 2,425 mg -NO_3 ion/L water Daily WI for group 3,325 mg $NaNO_3$/L = 0.209 L Average bw = 1.846 kg at the end of the experiment corresponds to 280 mg -NO_3 ion/kg bw per day	All turkeys receiving nitrate suffered significantly greater mortality than the control group	Enlargement of kidneys. No consistent findings were observed in other tissues examined (brain, intestine, liver, spleen)		Drinking water	Adams et al. (1969)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Japanese quail (*Coturnix coturnix* Japonica), 15 weeks of age, 4 groups with 13 quail chicks Duration: 1 week	0 or 5,000 mg -NO_2 ion (as $NaNO_2$)/kg feed Bw = 120–140 g Default DMI for chickens and hens = 12 g per day corresponds to 460 mg NO_2^-/kg bw per day	Depressed growth rates, a decrease in haemoglobin and an increase in MetHb levels. Female Japanese quail, either controls or nitrite treated, were observed to have less blood haemoglobin, with approximately a twofold increase in blood methaemoglobin than males from the equivalent treatments. Deposition of nitrite in eggs.	No pathology was investigated		Feed	Stoewsand, 1970
In series of 3 experiments: Quails of 1 to 7 days of age, 6 groups/experiment 4th experiment: Quails of 1 to 105 days of age, 6 groups	1,925 to 4,332 mg -NO_3 ion/L drinking water 0, 481, 962, 1,925, 2,888 and 3,851 mg -NO_3 ion/L of drinking water	Increased mortality Mortality of 0, 4.4, 8.9, 15.5, 27 and 100%	No pathological findings No pathology was investigated		Drinking water	Adams (1974)
Pheasant chickens, 14 days of age	0, 15 mg -NO_2 ion/L and 500 mg -NO_3 ion/L Average bw = 121 g and 117 g, respectively Daily WI = 0.129 and 0.135 L, respectively, corresponding to 16.0 mg -NO_2 ion/kg bw per day and to 576.9 mg -NO_3 ion/kg bw per day	Nitrite and nitrate adversely affected blood parameters (increased MetHb)	With the nitrite supplementation, non-specific dystrophic changes in liver and kidneys, and villus oedema of the small intestine were also observed. While, with the nitrate supplementation, hyperaemia of liver, kidneys and mucosa of the small intestine and multiplication of the eosinophilic granulocytes in the villus stroma were observed.		Drinking water	Strnad and Persin (1983)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Cockerels of 2 months of age, 15 chickens per treatment Duration: 28 days	1,700 mg $NaNO_2$/kg feed (0, 1,133 mg -NO_2 ion/kg feed and 3,063 mg -NO_3 ion/kg feed) corresponding to 68 mg -NO_2 ion/kg bw per day, and 183 mg -NO_3 ion/kg bw per day. All values calculated with default values for CONTAM (EFSA, 2019)	Nitrite supplementation adversely affected the performance of cockerels (total bw gain 210 vs. 65 g, increase of the heart relative weight 0.74 vs. 0.90 g/100 g bw, decrease of the bursa relative weight 0.32 vs. 0.20 g/100 g bw), as well as blood parameters (decrease of erythrocyte numbers 3.4 vs. 2.4 $\times$ 10^6/µl, increase of MetHb 1.1 vs. 25.6 % of total pigment, increase of glutamic-pyruvic transaminase 23 vs. 35 units/L, increase of creatinine 9 vs. 18 mg/L and increase of urea 200 vs. 300 mg/L) Nitrate supplementation also adversely affected the performance of cockerels (total bw gain 210 vs. 180 g, decrease of the bursa relative weight 0.32 vs. 0.25 g/100 g bw), as well as blood parameters (decrease of erythrocyte numbers 3.4 vs. 2.9 $\times$ 10^6/µl, and increase of MetHb 1.1 vs. 8.0 % of total pigment)	Increase of heart relative weight, decrease of bursa relative weight		Feed	Atef et al. (1991)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Chickens Duration: 35 days Turkeys Duration: 14 days	0, 200, 400, 800, 1,200, 1,600 mg NO_2 ion (as $NaNO_2$)/kg feed For chickens, the value of 1,600 mg -NO_2 ion/kg feed corresponds to 96 mg -NO_2 ion/kg bw per day Value calculated with default values for CONTAM (EFSA, 2019)	Reduction in growth rate and decrease in haemoglobin and an increase in MetHb levels for both species	No pathological findings (heart weight examined)		Feed	Diaz et al. (1995)
12 groups of Arbor Acres chickens for fattening were used in a 4×3 experimental design 90 one-day old birds/ treatment, allocated equally to two replicate pens Duration: 42 days	1.90, 2.72, 3.55 and 5.19 mg NO_3^-N/L drinking water (8.41, 12.05, 15.72 and 23.00 mg - NO_3^-/L drinking water with three water pH values (5.75, 6.25 and 6.75). Group 1.9 and 2.72 mg NO_3-N/L drinking water: Average bw for 3 pH treatments= 1855.5 g and 1818.1 g Average WI= 227.4 mL and 232 mL per day 8.41 and 12.05 mg - NO_3^-/L corresponds to 1 and 1.5 mg - NO_3^-/kg bw per day	Final body weight and relative thymus weight decreased with 12.05, 15.72 and 23.00 mg NO_3^-/L of drinking water vs. the control group of 8.41 mg NO_3^-/ L of drinking water	Relative thymus weight decreased. No differences were observed in weight of other tissues examined (bursa, liver, spleen)		Drinking water	Grizzle et al. (1996)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
One-day-old newly hatched commercial broiler (Cobb®) unsexed chicks. Chicks were wing-banded and randomly allocated to 2 treatments with three replicates (20 chicks each). Duration: 35 days	Levels 0 and 1,000 mg $NaNO_2$ ion/kg feed, equivalent to 0, and 667 mg -NO_2 ion/kg feed. Daily FI = 0.073 kg Average bw = 1,558 kg	The nitrite supplementation adversely affected the performance of chickens (total bw 1,714 vs. 1,597 g), as well as blood parameters (decrease of haemoglobin 17.90 vs. 15.04 g/100 mL, increase of methemoglobin 1.04 vs. 1.15 % of total pigment), while a higher death rate was also recorded (0 vs. 10%).	In the autopsy of dead chicken due to nitrite poisoning, dark brown blood, dehydration, mucoid enteritis, hyperaemia of the intestinal mucosa and petechial haemorrhages in the epicardium were seen		Feed	Bilal and Can Kutay (2002)
Broiler chickens, 1 to 42 days of age Duration: 42 days	Sodium nitrate (5.4 vs. 27.4 mg $NaNO_3$/L) Total WI = 3× FI = 3× 4.306 kg = 12.918 L Whole bw = 2.330k g The value of 27.4 mg $NaNO_3$/L corresponds to 20.0 mg NO_3^-/L, and to 2.7 mg NO_3^-/kg bw per day.	Nitrate negatively affected bw gain during finisher period (25–42 days) and feed to gain ratio during overall period. Nitrate also decreased blood uric acid, total antioxidant capacity and blood pO_2 and increased blood pCO_2.	No pathology was investigated		Drinking water	Akhavast and Daneshyar (2017)
15 Chickens (*Gallus gallus domesticus*) and 15 domestic mallard ducks (*Anas platyrhynchos domestica*)	Chickens: 0 to 175 mg $NaNO_2$/kg bw Ducks: 0 to 175 mg $NaNO_2$/kg bw	Both chickens and ducks displayed symptoms of methaemoglobinaemia including lethargy, shortness of breath, loss of co-ordination and loss of consciousness.	Necropsy of chickens and ducks that died found that all the birds appeared cyanotic – they were very pale with a bluish discolouration of the skin and mucous membranes. Their blood had a dark brown colouration attributed to methaemoglobinaemia induced by NaNO2	LD_{50} value for $NaNO_2$ in solution approximately 68.50 mg $NaNO_2$ (45.5 mg -NO_2 ion)/kg bw for both chickens and ducks	Oral gavage	Shapiro et al. (2017)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Chickens, domestic mallard ducks, pigeons (*Columba livia f. domestica*), budgerigars (*Melopsittacus undulates*) and wētā (Family: Rhaphidophoridae)	Chickens: 0 to 1302.93 mg $NaNO_2$ eaten (mg/kg bw) Ducks: 0 to 99.3 mg $NaNO_2$ eaten (mg/kg bw)	4 out of 6 chickens eating the bait died. Chickens displayed the same symptoms of methaemoglobinaemia as observed in the oral gavage trials. 1 out of 4 ducks eating the bait died. The duck that consumed 1.3 g of NaNO2 paste bait displayed the same symptoms of methaemoglobinaemia as the gavage trials and died after consuming approximately 99.3 mg/kg of $NaNO_2$.	Findings of the necropsies of the four birds that died were identical to those from chickens that died in the oral gavage trial. The necropsy of the one bird that died was identical to that for birds that died in oral gavage trials.	Toxic paste LD_{50} value for chickens approximately 254.6 mg $NaNO_2$ (169.7 mg -NO_2 ion)/kg bw	Free-feeding trials	
Horse						
9 mares (5 pregnant) Duration: 24 h	Nitrate levels in pasture grass and alfalfa ranged from 400 to 9,923 mg/kg DM and 2,232 to 4,341 mg/kg DM diet, respectively Total consumption of nitrate was estimated to approximately 80 g per animal Bw default values for CONTAM (EFSA, 2019)	Clinical findings developed over a short period and included severe abdominal pain, limited response to analgesics and antispasmodics, diarrhoea, shallow and rapid breathing, tachycardia, blue-brown discoloration of the mucosal membranes, tremors, ataxia, convulsions before death and abortion in the pregnant mares	The main post mortem finding was chocolate brown-coloured blood as well as congestion and inflammation of the intra-abdominal organs. Uterine rupture, colonic ruptures and torsio uteri were seen in the pregnant mares. Stomach and intestinal ruptures were seen in four non-pregnant mares		Ingestion of forage and alfalfa	Oruc et al. (2010)
Dogs						
No study design (Book Chapter) 2 dogs Duration: 105 and 125 days	20,000 mg $NaNO_3$ (14,588 mg NO_3^-)/kg feed, corresponding to 210 mg NO_3^-/kg bw per day Value calculated with default values for CONTAM (EFSA, 2019)	No observed adverse effects or changes in blood parameters	No pathology was investigated		Feed	Lehman (1958)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Beagle dogs (1 male and 6 females per group) and their offspring Duration: 16 months	0, 300, 600 or 1,000 mg $NaNO_3$/L of drinking water (0, 218.8, 437.6 and 729.4 mg - NO_3^-/L of drinking water. All values calculated with default values from EFSA, 2019 The value of 729.4 mg NO_3^-/L water corresponds to 459.5 mg NO_3^-/dog per day, and to 30.6 mg NO_3^-/kg bw per day	No clinical manifestation of hypothyroidism (blood thyroxin and triiodo-thyronine concentrations determined) was observed in any of the adult or puppy Beagles	No pathology was investigated		Drinking water	Kelley et al. (1974)
Dogs	Oral exposure, gavage: 35–71 mg NO_2^- /kg bw per day 53–71 mg NO_2^-/kg bw per day Chronic oral exposure: 89 mg NO_2^- /kg bw per day	Increase in MetHb Deaths Death after 133–313 h.	Chocolate-brown colour of blood and tissues, general stasis, oedema, catarrhal inflammation, superficial focal necrosis of the mucous membrane of the alimentary tract, protein degeneration of the parenchymal organs with necrotic lesions in the liver and kidneys, vacuolisation of the vascular net of the glomerules and mononuclear infiltration of the kidneys		Oral and subcutaneous exposure	Michalski (1963)
56 Beagle dogs, 4 dose groups with 7 male and 7 female dogs per treatment, approximately 15 months old Duration: 26 weeks	0, 7, 14 and 28 mg $NaNO_2$ (0, 4.67, 9.33 and 18.67 mg NO_2^-)/kg bw per day.	Cyanosis and MetHb, primarily at the high dose group	No pathology was investigated	Intravenous NOAEL: 9.33 mg -NO_2 ion/kg bw per day	Intravenous administration	Tepper et al. (2014)

Study design Breed, age, gender, exposure period, animal weight	Doses or feed concentrations	Clinical signs/biochemical changes	Pathological findings	NOAEL/LOAEL and endpoint	Remarks	Reference
Cats						
3 cats	On average 2,850 mg nitrite/kg canned feed (1,900 mg -NO_2 ion/kg canned feed)	Acute toxicosis with death due to extensive MetHb formation	Brownish discolouration of the blood, pale mucous membranes, haemorrhage in lung lobes and dorsal surface of tongue, enlarged liver with centrilobular congestion and peripheral fatty change		Canned feed	Worth et al. (1997)
No study design (Book Chapter) 1 cat Duration: 105 days	Oral dose 4,100 mg $NaNO_2$ (2,733 mg -NO_2 ion)/cat/105 days, corresponding to 6.5 mg -NO_2 ion/kg bw per day Value calculated with default values for CONTAM (EFSA, 2019)	No adverse effects were observed on growth performance and organ weight	No effects on weight of important organs was noted		Feed	Lehman (1958)

Appendix B – Benchmark Dose Modelling

B.I. Report using the BMDS Version 2.7.0.4

Data Description

The CONTAM Panel identified a study by Benu et al. (2016) as the critical study. The authors measured the MetHb content of blood in 12 *Bos indicus* steers. Animals were exposed to daily nitrate dose (0, 30, 40 or 50 g NO_3^-/day) for 7 days. Blood samples were collected every 2 h starting at 06.00 h for a period of 7 days. The MetHb content in the blood of steers increased dose-dependently with nitrate dose. The CONTAM Panel used the maximum MetHb content measured at each of the exposure days 1,2,3,4, 5, 6 and 7, as relevant dose metric for BMD calculations.

The calculation of the BMD, the $BMDL_{10}$ and the BMDU at each sampling day is presented in Appendix B. The lowest $BMDL_{10}$ of 20.26 g/day was chosen, corresponding to the exponential model 5 at day 2.

For the sake of brevity, in this report, the full data set is presented only for day 2.

Selection of the BMR

The CONTAM Panel chose a benchmark response (BMR) of 10% (MetHb concentrations in %), considering that the critical adverse effect of hypoxia can occur at levels above this value (Benu et al., 2016, 2018; Benu, 2017).

For technical reasons, it was not possible to use the EFSA web tool for BMD analysis, which uses the R-package PROAST. In PROAST, it is not possible to define a cut-off point (such as the 10% of MetHb) as the BMR but only the relative change over the measured background of the data used. The dose-response analysis and the calculation of the BMD and the $BMDL_{10}$, i.e. its 95% lower confidence limit was therefore performed using BMDS Version 2.7.0.4 (US EPA, 2017).

Specification of Deviations from Default Assumptions

General assumptions

Differences between BMDS and PROAST exist and are presented in the EFSA guidance (EFSA Scientific Committee, 2017). The most important of them are summarised below:

- in BMDS, the variance can be either specified as constant or it can be modelled as a function of the mean response, while PROAST always assumes the variance to be constant for continuous data.
- data are assumed to be normally distributed in BMDS while they are assumed to be log-normally distributed in PROAST.
- in BMDS, the variance can be either specified as constant or it can be modelled as a function of the mean response, while PROAST always assumes the variance to be constant for continuous data.
- In PROAST, only two exponential models are available (models 3 and 5), while BMDS provides a number of nested family of exponential models of 5, and also includes power, linear and polynomial models (these models were not used in this Opinion).

Dose-response models

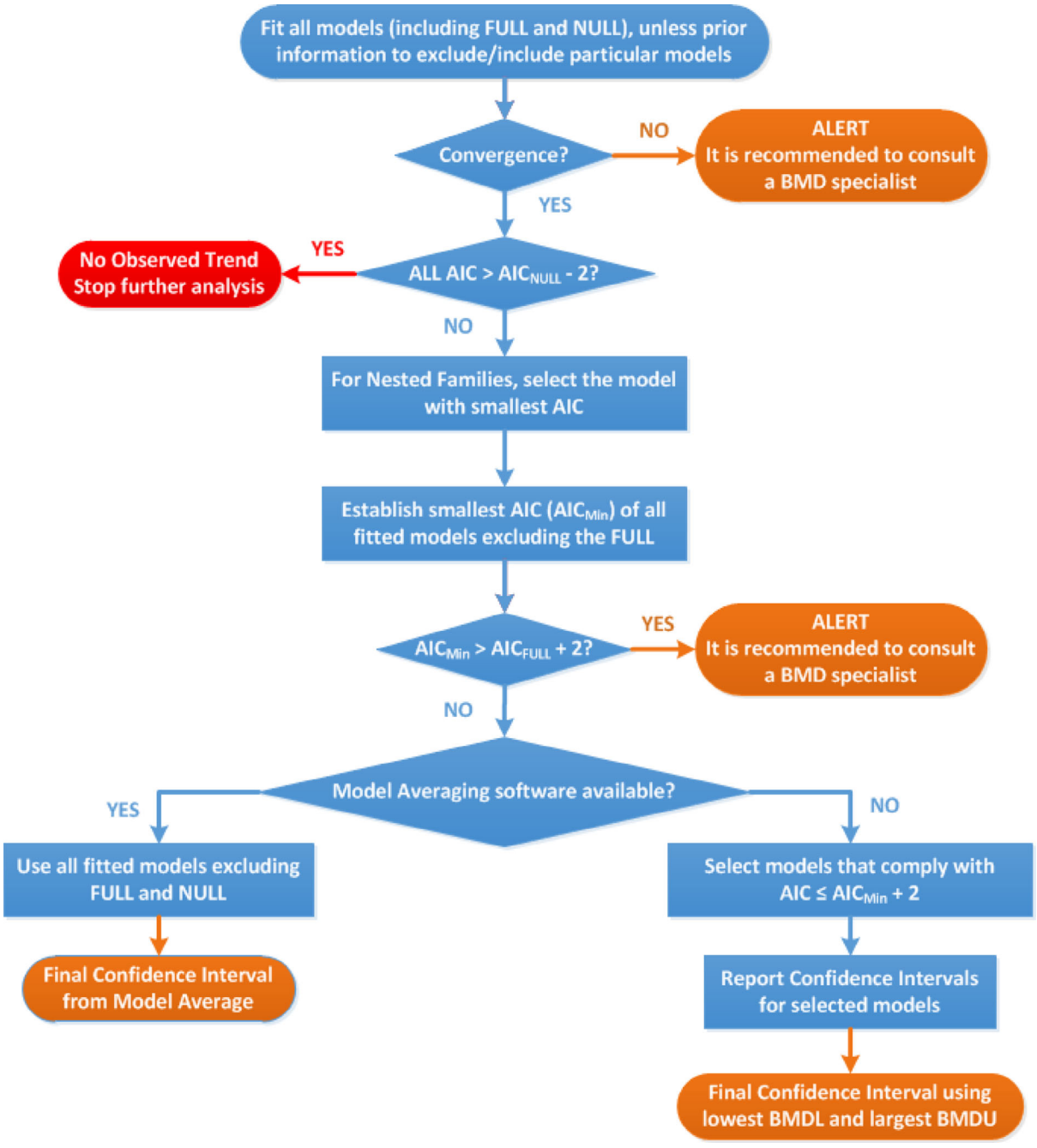
Default set of fitted models:

Model	Number of parameters	Formula
Null	1	$y = a$
Full	No. of groups	μ = group mean
Exp model 3	4	$\mu(X) = \gamma + e^{(kX)^d}$
Exp model 5	6	$\mu(X) = \gamma(c - (c-1)e^{-(kX)^d})$
Hill model	5	$\mu(X) = \gamma + \nu\frac{X^\eta}{k^\eta + X^\eta}$

Procedure for the selection of $BMDL_{10}$

The CONTAM panel evaluated the dose-response information with the models available in EPA's Benchmark Dose Software and followed the default assumptions from the EFSA Scientific Committee (2017):

- the models were fitted to the data assuming a log-normal distribution and a constant variance.
- the lowest $BMDL_{10}$ was chosen among the Hill model and exponential models (3 and 5), to be consistent with the EFSA Scientific Committee (2017). Results from other model (linear, power, exponential 2, and 4) were not considered.



Flowchart for selection of $BMDL_{10}$

Results

Response variable:

% MetHb in the blood

Fitted Models

Model	Converged	loglik	npar	AIC
full model	Yes	4.829062	5	0.3418751
null model	Yes	−10.34451	2	24.68902
Expon. M3-	Yes	2.126146	4	1.747707
Expon. M5-	Yes	2.576706	5	4.846589
Model	**Converged**	**loglik**	**npar**	**AIC**
full model	Yes	−36.422622	5	82.845243
null model	Yes	−42.678272	2	89.356544
Hill	Yes	−36.683541	5	81.367083

Note: In BMDS, the dose-response with the Hill model can be analysed only with normal distribution, which explains the difference between AIC for the full and null model with results from exponential model.

Weights for Model Averaging

Not possible with BMDS for continuous data

Final BMD Values

Exponential model

Endpoint	subgroup	$BMDL_{10}$	BMDU
Mean		20.2565	33.5199

Hill model

Endpoint	subgroup	$BMDL_{10}$	BMDU
Mean		5.00E-14	34.5286

Estimated Model Parameters

Exponential model

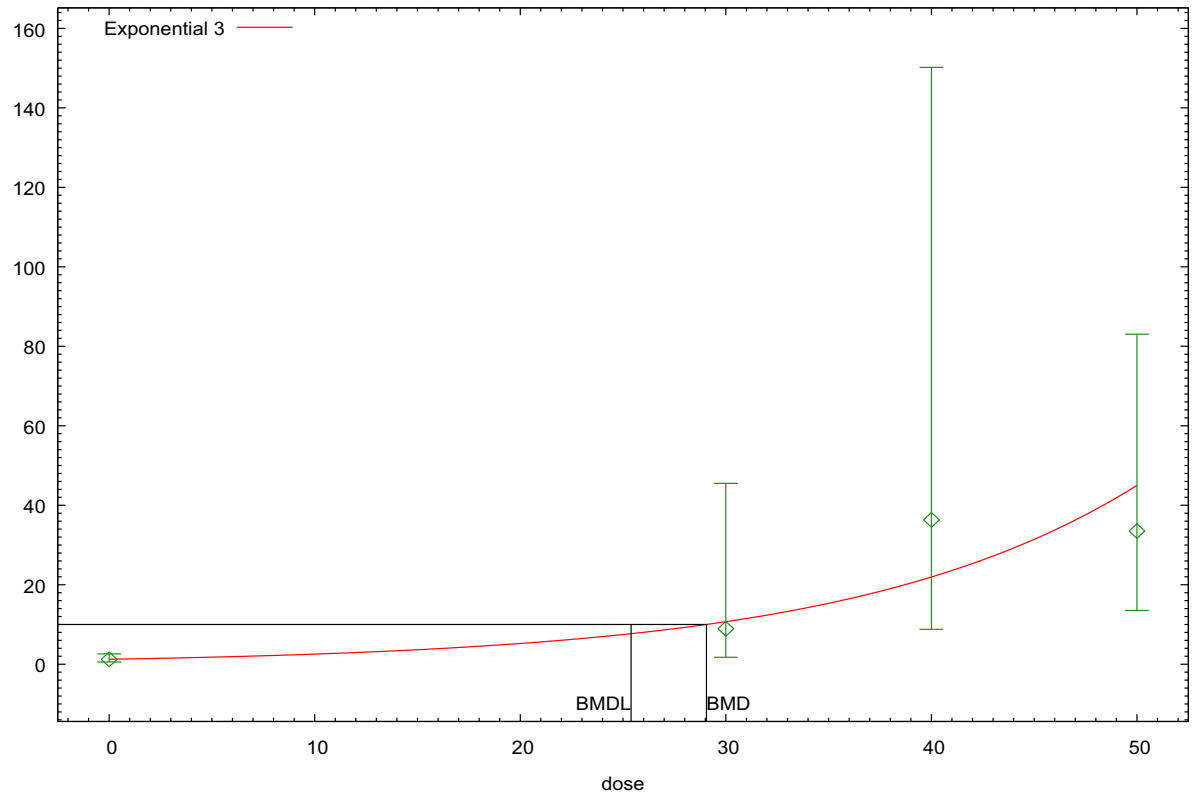
Variable	Model 3	Model 5
alpha	−1.35436	−1.42945
rho	0	0
a	1.24126	1.19885
b	0.0718079	0.0010282
c	–	156411
d	1	2.83484

Hill Model

Parameter estimates				
Variable	Estimate	95.0% Wald Confidence Interval		
		SE	Lower conf. limit	Upper conf. limit
Alpha	166.32	67.9005	33.2391	299.404
Intercept	1.1123	7.33949	−13.2728	15.4974
v	38.4193	9.00588	20.7681	56.0705
n	18	NA		
k	31.7348	2.11775	27.5841	35.8855

Visualisation

Exponential 3 Model, with Point Estimate BMR of 10 for the BMD and 0.95 Lower Confidence Limit for the BMDL



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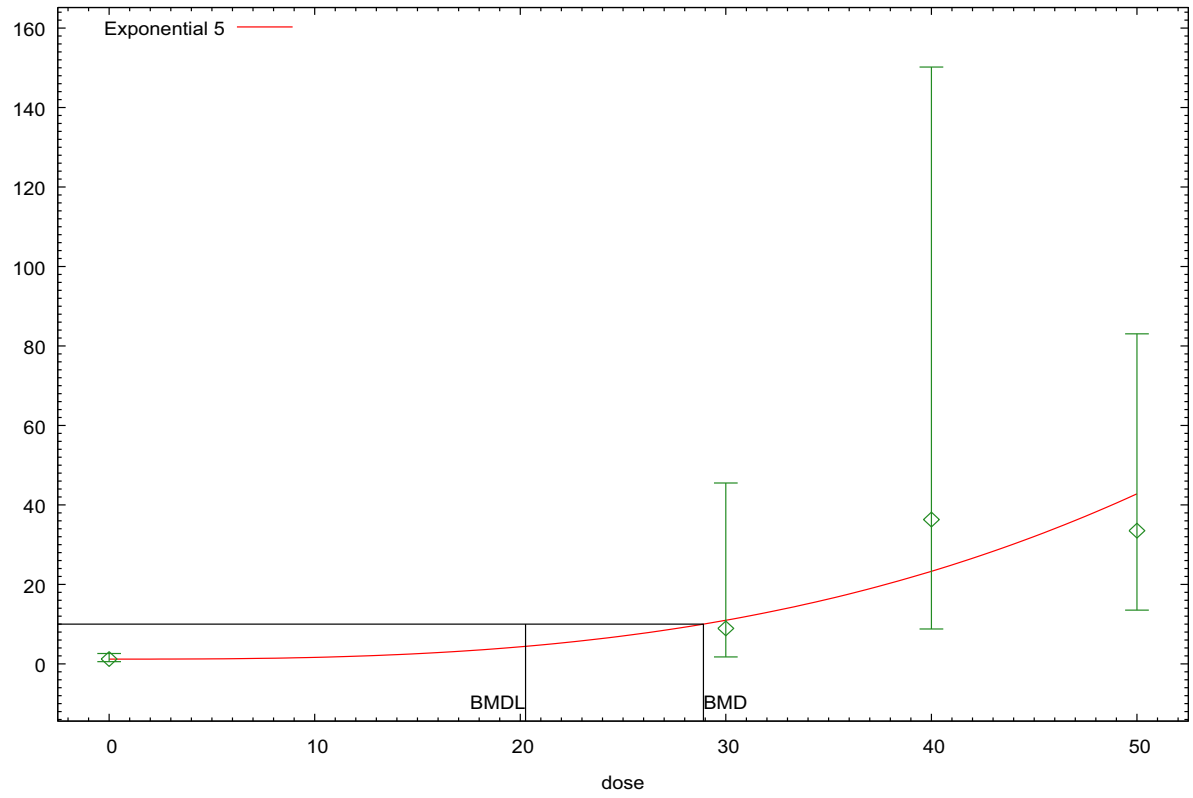
Exponential 5 Model, with Point Estimate BMR of 10 for the BMD and 0.95 Lower Confidence Limit for the BMDL



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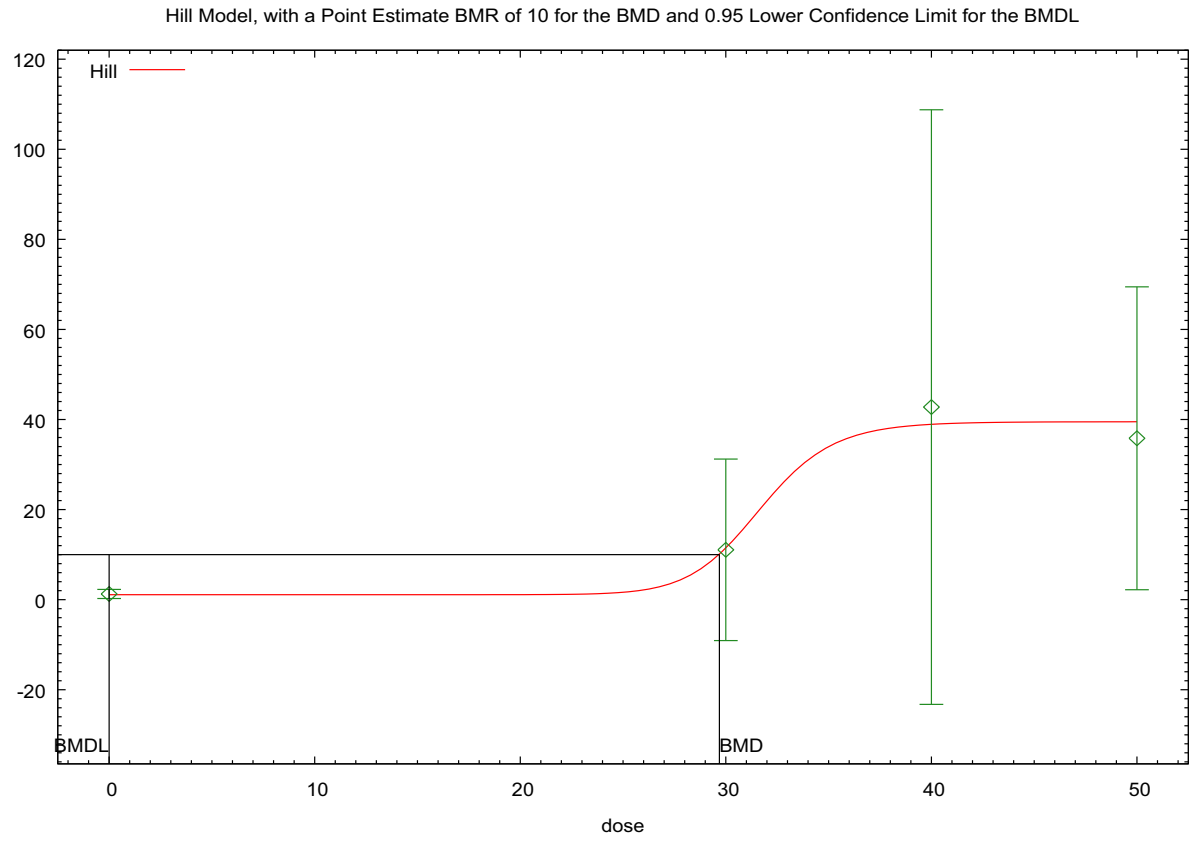


Conclusions

Results of the Hill model lead to unrealistic values; therefore, only two BMDL values from exponential model 3 and 5 are available. The Panel decided to deviate from the EFSA guidance (EFSA Scientific Committee, 2017) by not selecting the model resulted in the lowest AIC but the one with the lowest $BMDL_{10}$ of 20.26 g nitrate/per day of exponential model 5 on day 2, as shown in table below 'comparative results from BMD analysis on each day.

B.I.1. Data reported for each day

Data reported for day 1

Dose	N	Mean	SD
0	3	1	0.60827625
30	3	6.93333333	6.57596634
40	3	24.7333333	19.8021043
50	3	28.5333333	22.2372061

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Data reported for day 2

Dose	N	Mean	SD
0	3	1.26666667	0.40414519
30	3	11.0666667	8.11315804
40	3	42.7666667	26.5748628
50	3	35.8333333	13.5371095

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Data reported for day 3

Dose	N	Mean	SD
0	2	1.55	0.07071068
30	3	10.7333333	7.82133833
40	3	20.8	30.4320555
50	3	41.9333333	22.0912502

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Data reported for day 4

Dose	N	Mean	SD
0	3	1.33333333	0.89628864
30	3	11.0333333	6.49332991
40	2	53.65	33.3047294
50	3	52.5333333	23.1698799

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Data reported for day 5

Dose	N	Mean	SD
0	3	1.83333333	1.00166528
30	3	10.2333333	6.76042405
40	2	48.15	41.7900108
50	3	51.1333333	17.2163682

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Data reported for day 6

Dose	N	Mean	SD
0	3	1.2	0.81853528
30	3	8.43333333	5.3379147
40	2	46.9	34.931075
50	3	56.8333333	19.9580393

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Data reported for day 7

Dose	N	Mean	SD
0	3	1.1	0.7
30	3	9.9	2.65141472
40	2	59.55	22.1324423
50	3	59.4	17.4364561

Dose, in g
N, number of animals
Mean, % of MetHb
Sdt, standard deviation

Comparative results from BMD analysis on each day

	Model Name	BMD	$BMDL_{10}$	BMDU	AIC	AIC Null	AIC Full
Day 1	Exponential3	36.351	31.5745	41.4708	8.442033	24.60651	8.366377
	Exponential5	36.0132	29.3118	40.622	9.951082		
	Hill	31.6579	5E-14	38.8727	82.524069	84.025668	82.524069
Day 2	Exponential3	29.0561	25.3917	33.5199	1.747707	24.689	0.34187
	Exponential5	28.9091	20.2565	33.3431	4.846589		
	Hill	29.6868	5.00E-14	34.5286	81.367083	89.356544	82.845243
Day 3	Exponential3	34.3632	27.257	39.6693	7.722574	19.03872	8.812637
	Exponential5	33.6897	24.0981	38.6598	10.15077		
	Hill	30.1586	5E-14	44.9038	82.528092	83.021630	82.510622
Day 4	Exponential3	27.9545	5E-09	33.0908	2.275606	25.46242	2.367981
	Exponential5	28.9846	22.2971	32.9325	5.525275		
	Hill	29.7463	5.007E-14	34.7234	77.651982	87.998020	79.620343
Day 5	Exponential3	29.6187	23.941	34.3756	3.061733	22.99288	2.373632
	Exponential5	30.86	27.686	33.1003	2.373638		
	Hill	29.932	5E-14	35.7095	80.38714	87.213363	80.387140
Day 6	Exponential3	31.3945	26.5035	35.4599	4.036333	26.19913	2.84615
	Exponential5	31.6308	29.3325	33.7013	2.846207		
	Hill	30.5935	5.0253E-14	36.1222	78.638846	88.235589	78.638846
Day 7	Exponential3	27.6262	24.7938	31.977	–0.4166464	27.05044	–4.196642
	Exponential5	28.848	24.0634	32.0328	2.286692		
	Hill	29.9844	5E-14	33.3432	69.829632	88.983868	71.785566

B.II. Report using the R-package PROAST

- **Data Description**

The CONTAM Panel identified a study by Benu et al. (2016) as the critical study. The authors measured the MetHb content of blood in 12 *Bos indicus* steers. Animals were exposed to daily nitrate dose (0, 30, 40 or 50 g NO_3^-/day) for 7 days. Blood samples were collected every 2 h starting at 06.00 h for a period of 7 days. The MetHb content in the blood of steers increased dose-dependently with nitrate dose. The CONTAM Panel used the maximum MetHb content measured at each of the exposure days 1,2,3,4, 5, 6 and 7, as relevant dose metric for BMD calculations. Data used for analysis are shown in the Appendix.

- **Selection of the BMR**

The BMR cut-off value of 10% increase of MetHb has been converted to a percentage change compared to the background response (as defined in the EFSA guidance) by back-calculating a threshold level from the definition of continuous BMR given in PROAST. This is:

$$BMR = (F(BMD) - F(0))/F(0)$$

where F(BMD) is the change in the level of the effect (in this case, the selected threshold level of 10% of methaemoglobin in the blood) at the benchmark dose and F(0) is the level observed in the control (in this case, the higher level observed at the control; 1.83%, day 5). This corresponds approximately to a BMR of 450%.

A 90% confidence interval around the BMD will be estimated, the lower bound is reported by BMDL and the upper bound by BMDU.

- **Software Used**

Results are obtained using the EFSA web-tool for BMD analysis, which uses the R-package PROAST, version 69.0, for the underlying calculations.

- **Specification of Deviations from Default Assumptions**

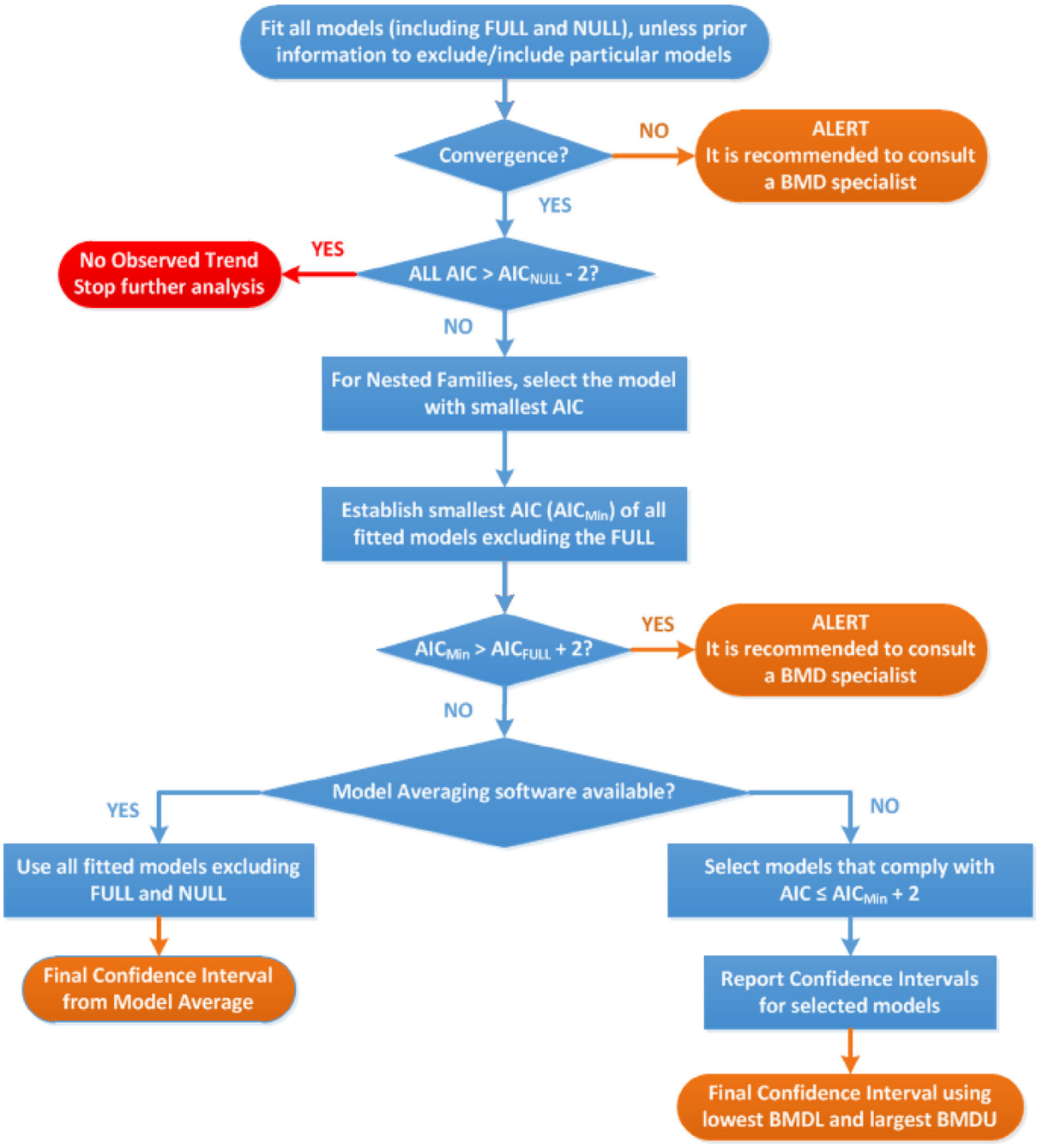
Dose-response models

Default set of fitted models:

Model	Number of parameters	Formula
Null	1	$y = a$
Full	No. of groups	$y =$ group mean
Exp model 3	3	$y = a \cdot \exp(bx^d)$
Exp model 4	4	$y = a \cdot (c - (c-1)\exp(-bx^d))$
Hill model 3	3	$y = a \cdot \left(1 - \frac{x^d}{b^d + x^d}\right)$
Hill model 4	4	$y = a \cdot \left(1 - \frac{(c-1) \cdot x^d}{b^d + x^d}\right)$
Inverse Exponential	4	$y = a \cdot (1 + (c-1)\exp(-bx^{-d}))$
Log-Normal Family	4	$y = a \cdot (1 + (c-1)\phi(\ln b + d\ln x))$

As a covariate is included in the analysis, these models will also be fitted assuming that some of the parameters [background response parameter (a), potency parameter (BMD) and/or variance (var)] depend on the subgroup defined by the covariate. Therefore, the number of parameters in each model might be larger than indicated in the table above.

Procedure for selection of BMDL



Flowchart for selection of BMDL

Results

- **Response variable: mean**
- **Fitted Models**

Model	Converged	loglik	npar	AIC
Full	NA	NA	NA	NA
Null model	Yes	–145.16	2	294.32
Null odel-a	Yes	–144.59	8	305.18
Expon. m3-	Yes	–70.97	4	149.94

Model	Converged	loglik	npar	AIC
Expon. m3-a	Yes	−65.49	10	150.98
Expon. m3-b	Yes	−64.20	10	148.40
Expon. m3-ab	Yes	−62.46	16	156.92
Expon. m5-	Yes	−98.39	5	206.78
Expon. m5-a	Yes	−60.16	11	142.32
Expon. m5-b	Yes	−60.58	11	143.16
Expon. m5-ab	Yes	−57.52	17	149.04
Expon. m5-av	Yes	−56.46	17	146.92
Hill m3-	Yes	−70.90	4	149.80
Hill m3-a	Yes	−65.41	10	150.82
Hill m3-b	Yes	−64.11	10	148.22
Hill m3-ab	Yes	−62.35	16	156.70
Hill m5-	Yes	−67.64	5	145.28
Hill m5-a	Yes	−61.24	11	144.48
Hill m5-b	Yes	−61.06	11	144.12
Hill m5-ab	Yes	−58.95	17	151.90
Hill m5-bv	Yes	−58.72	17	151.44
Inv.Expon. m3-	Yes	−70.37	4	148.74
Inv.Expon. m3-a	Yes	−64.77	10	149.54
Inv.Expon. m3-b	Yes	−63.40	10	146.80
Inv.Expon. m3-ab	Yes	−61.55	16	155.10
Inv.Expon. m5-	Yes	−66.98	5	143.96
Inv.Expon. m5-a	Yes	−60.16	11	142.32
Inv.Expon. m5-b	Yes	−61.40	11	144.80
Inv.Expon. m5-ab	Yes	−59.68	17	153.36
Inv.Expon. m5-av	Yes	−56.24	17	146.48
LN m3-	Yes	−70.63	4	149.26
LN m3-a	Yes	−65.08	10	150.16
LN m3-b	Yes	−63.73	10	147.46
LN m3-ab	Yes	−61.92	16	155.84
LN m5-	Yes	−66.98	5	143.96
LN m5-a	Yes	−60.16	11	142.32
LN m5-b	Yes	−61.09	11	144.18
LN m5-ab	Yes	−59.11	17	152.22
LN m5-av	Yes	−56.24	17	146.48

- **Estimated Model Parameters**

EXP

estimate for var- : 0.2685
estimate for a-1 : 0.7165
estimate for a-2 : 1.168
estimate for a-3 : 0.9125
estimate for a-4 : 1.313
estimate for a-5 : 1.36
estimate for a-6 : 1.173
estimate for a-7 : 1.365
estimate for CED- : 28.4
estimate for c- : 37.25
estimate for d- : 3.864

HILL

estimate for var- : 0.2747
estimate for a- : 1.136
estimate for CED-1 : 32.14
estimate for CED-2 : 27.24
estimate for CED-3 : 30.78
estimate for CED-4 : 26.17
estimate for CED-5 : 27.04
estimate for CED-6 : 27.5
estimate for CED-7 : 25.51
estimate for c- : 74.22
estimate for d- : 3.863

INVEXP

estimate for var- : 0.2685
estimate for a-1 : 0.7165
estimate for a-2 : 1.168
estimate for a-3 : 0.9125
estimate for a-4 : 1.313
estimate for a-5 : 1.36
estimate for a-6 : 1.173
estimate for a-7 : 1.365
estimate for CED- : 28.91
estimate for c- : 42.26
estimate for d- : 5.544

LOGN

estimate for var- : 0.2685
estimate for a-1 : 0.7165
estimate for a-2 : 1.168
estimate for a-3 : 0.9125
estimate for a-4 : 1.313
estimate for a-5 : 1.36
estimate for a-6 : 1.173
estimate for a-7 : 1.365
estimate for CED- : 28.69
estimate for c- : 38.78
estimate for d- : 4.088

- **Weights for Model Averaging**

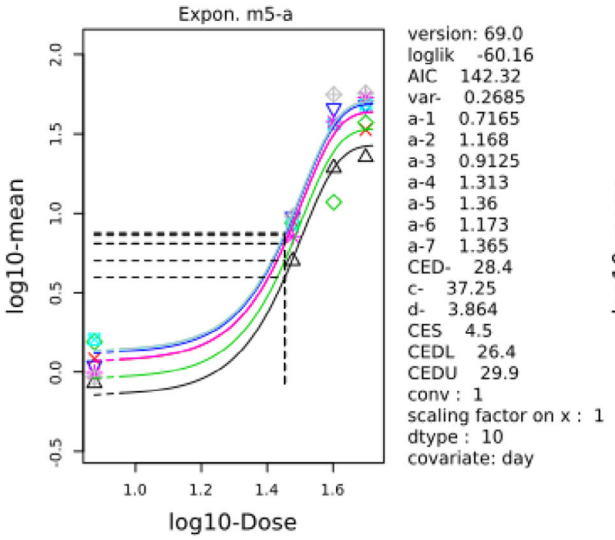
EXP	HILL	INVEXP	LOGN
0.29	0.12	0.29	0.29

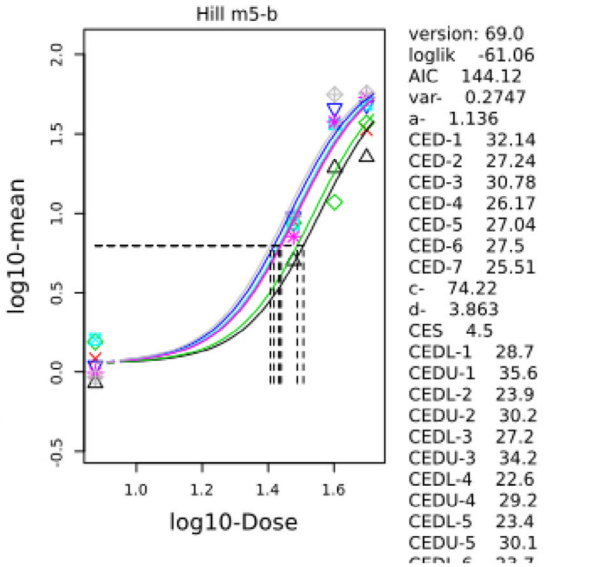
- **Final BMD Values**

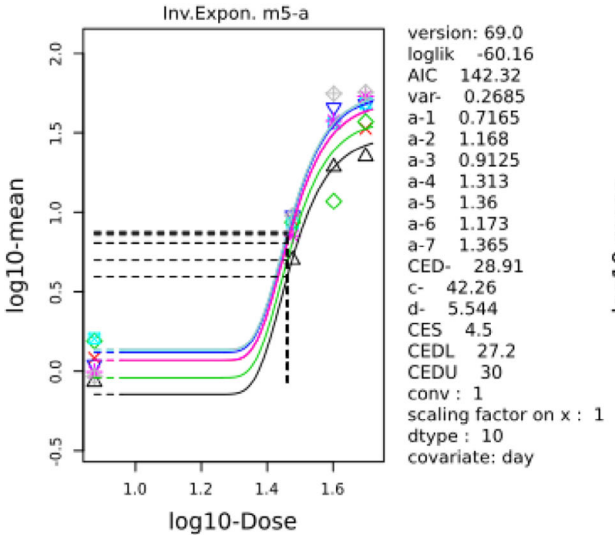
Endpoint	Subgroup	BMDL	BMDU
Mean	1	25.7	33.8
Mean	2	25.0	30.6
Mean	3	25.1	32.0
Mean	4	24.3	30.2
Mean	5	24.8	30.2
Mean	6	25.1	31.2
Mean	7	24.2	30.3

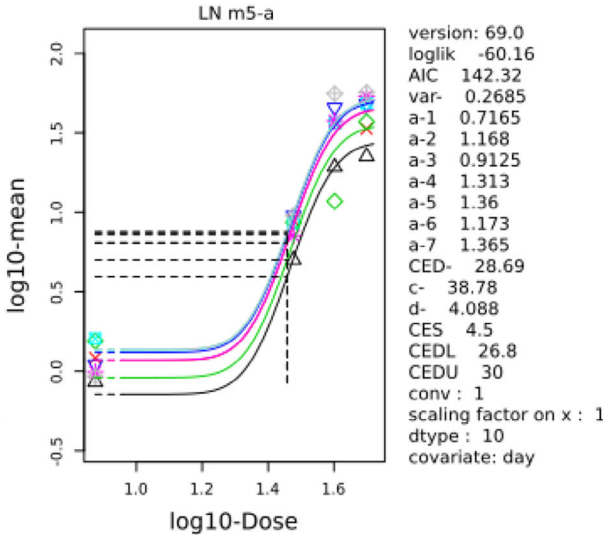
Confidence intervals for the BMD are based on 200 bootstrap data sets.

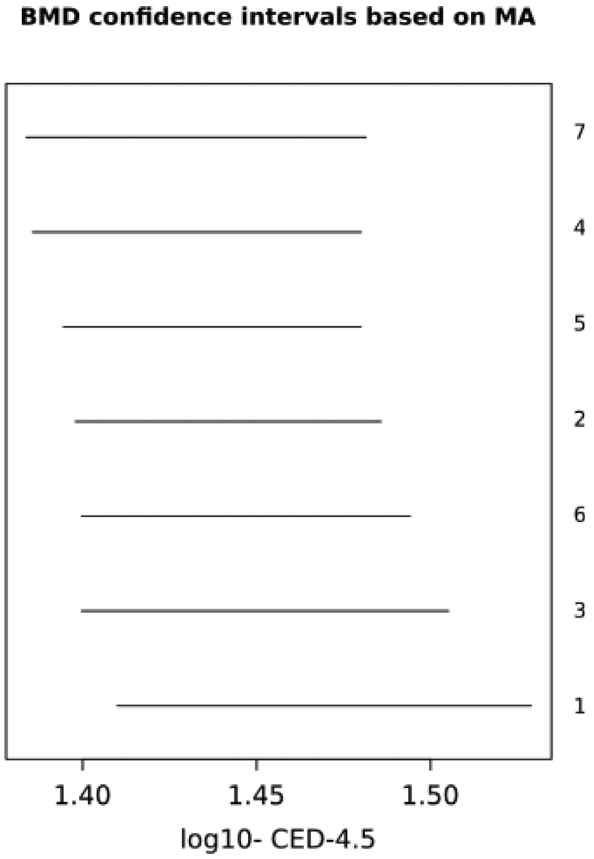
- **Visualisation**

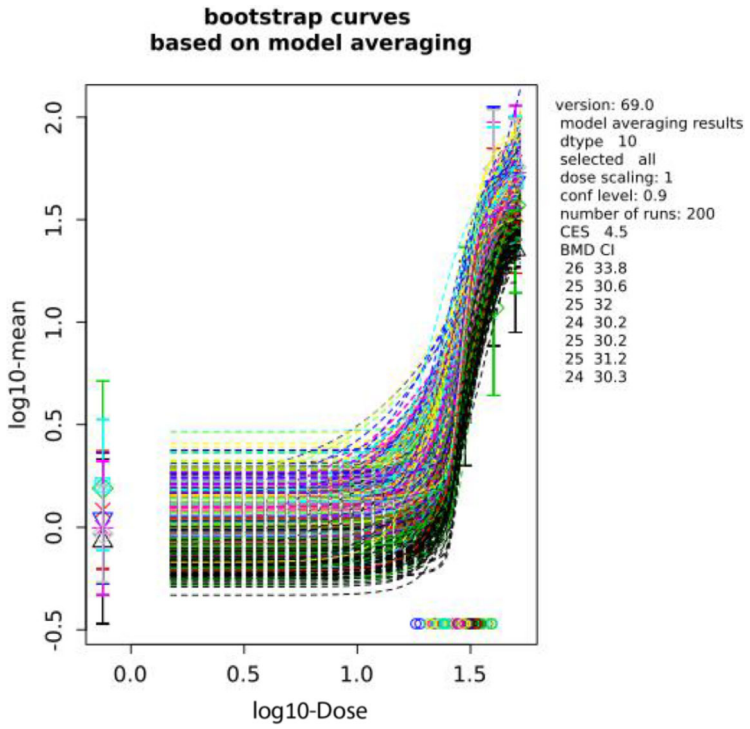









BMD confidence intervals based on MA
7
4
5
2
6
3
1
1.40
1.45
1.50
log10- CED-4.5


bootstrap curves
based on model averaging
log10-mean
log10-Dose
version: 69.0
model averaging results
dtype 10
selected all
dose scaling: 1
conf level: 0.9
number of runs: 200
CES 4.5
BMD CI
26 33.8
25 30.6
25 32
24 30.2
25 30.2
25 31.2
24 30.3

Appendix

Data used for analysis:

Dose	Mean	SD	N	Day
0	1.00	0.61	3	1
30	6.93	6.58	3	1
40	24.73	19.80	3	1
50	28.53	22.24	3	1
0	1.27	0.40	3	2
30	11.07	8.11	3	2
40	42.77	26.57	3	2
50	35.83	13.54	3	2
0	1.55	0.07	2	3
30	10.73	7.82	3	3
40	20.80	30.43	3	3
50	41.93	22.09	3	3
0	1.33	0.90	3	4
30	11.03	6.49	3	4
40	53.65	33.30	2	4
50	52.53	23.17	3	4
0	1.83	1.00	3	5
30	10.23	6.76	3	5
40	48.15	41.79	2	5
50	51.13	17.22	3	5
0	1.20	0.82	3	6
30	8.43	5.34	3	6
40	46.90	34.93	2	6
50	56.83	19.96	3	6
0	1.10	0.70	3	7
30	9.90	2.65	3	7
40	59.55	22.13	2	7
50	59.40	17.44	3	7

Annex I – Literature search and selection for relevance of studies related to the toxicity of nitrate and nitrite in feed

Data and methodologies

Data

To search for different types of publications providing information on nitrate and nitrite, the following databases were used:

Web of Science Core Collection	http://webofknowledge.com/WOS
PubMed	http://www.ncbi.nlm.nih.gov/pubmed

Methodologies

Date of search

7/5/2019–Last update 13/2/2020

Search protocol and search strategies

For full transparency and reproducibility, all searches performed are recorded in search protocols, including search queries used in individual databases to retrieve potentially relevant studies for subsequent screening of titles and abstracts.

Search terms

PubMed

Date of the search: 7/5/2019 and 13/2/2020

Search	Query	Items found
#11	Search #9 NOT #10	3,914
#10	Search (salami[tiab] OR ham[tiab] OR curing[tiab] OR cured[tiab] OR jambon[tiab] OR cytometry[tiab] OR manure[tiab] OR leach*[tiab] OR sludge[tiab] OR "Processed meat"[tiab] OR "red meat"[tiab] OR "self life"[tiab] OR rat[tiab] OR rats[tiab] OR mice[tiab] OR mouse[tiab] OR monkey[tiab] OR monkeys[tiab] OR zebrafish[tiab] OR medaka[tiab] OR "human milk"[tiab] OR "humans"[Mesh] OR human[ti] OR humans [ti]) Filters: Publication date from 1995/01/01; English	20,523,816
#9	Search #8 Filters: Publication date from 1950/01/01; English	6,037
#8	Search #7 Filters: Publication date from 1950/01/01	6,477
#7	Search #5 AND #6	6,493
#6	Search "Animals, Domestic"[Mesh] OR domestic animal*[tiab] OR "Ruminants"[Mesh] OR ruminat*[tiab] OR cattle[tiab] OR cow[tiab] OR cows[tiab] OR bull[tiab] OR bulls [tiab] OR calf[tiab] OR calves[tiab] OR heifer*[tiab] OR bullock*[tiab] OR veal[tiab] OR veal*[tiab] OR dairy herd*[tiab] OR dairy breed*[tiab] OR bovine*[tiab] OR ovis [tiab] OR ovine[tiab] OR ewe[tiab] OR ewes[tiab] OR lamb[tiab] OR lambs[tiab] OR sheep*[tiab] OR goat[tiab] OR goats[tiab] OR capra[tiab] OR capras[tiab] OR caprin*[tiab] OR buffalo*[tiab] OR bubalus[tiab] OR farm animal*[tiab] OR "Swine"[Mesh] OR swine[tiab] OR "sus scrofa "[tiab] OR "sus domestica"[tiab] OR "sus domesticus"[tiab] OR porcine[tiab] OR suidae[tiab] OR pig[tiab] OR pigs[tiab] OR piglet*[tiab] OR sow[tiab] OR sows[tiab] OR barrow*[tiab] OR boar[tiab] OR boars[tiab] OR hog[tiab] OR hogs[tiab] OR gilt[tiab] OR gilts[tiab] OR "Poultry"[Mesh] OR poultry[tiab] OR Poultries[tiab] OR "domestic bird"[tiab] OR "domestic birds"[tiab] OR "domesticated bird"[tiab] OR "domesticated birds"[tiab] OR fowl[tiab] OR fowls[tiab] OR galliform*[tiab] OR wildfowl*[tiab] OR gallinaceous bird [tiab] OR landfowl[tiab] OR chicken*[tiab] OR "Gallus gallus"[tiab] OR "Gallus domesticus"[tiab] OR broiler*[tiab] OR capon[tiab] OR capons[tiab] OR cockerel*[tiab] OR hen[tiab] OR hens[tiab] OR pullet[tiab] OR pullets[tiab] OR rooster[tiab] OR roosters[tiab] OR waterfowl*[tiab] OR Anatidae[tiab] OR duck [tiab] OR ducks[tiab] OR mallard*[tiab] OR "Anas platyrhynchos"[tiab] OR Geese	2,239,967

Search	Query	Items found
	[tiab] OR goose[tiab] OR anser[tiab] OR branta[tiab] OR Coturnix[tiab] OR quail* [tiab] OR (turkey[tiab] AND (animal*[tiab] OR "Animals"[Mesh])) OR Turkeys[tiab] OR meleagris[tiab] OR pigeon*[tiab] OR dove[tiab] OR doves[tiab] OR columb*[tiab] OR ostrich[tiab] OR ostriches[tiab] OR Struthio[tiab] OR "Equidae"[Mesh] OR equidae*[tiab] OR equus[tiab] OR horse*[tiab] OR equine*[tiab] OR colt[tiab] OR colts[tiab] OR foal[tiab] OR foals[tiab] OR yearling*[tiab] OR gelding*[tiab] OR mare [tiab] OR mares[tiab] OR pony[tiab] OR ponies[tiab] OR stallion*[tiab] OR filly[tiab] OR fillies[tiab] OR ass[tiab] OR asses[tiab] OR mule[tiab] OR mules[tiab] OR donkey* [tiab] OR pets[tiab] OR pet[tiab] OR "Dogs"[Mesh] OR dog[tiab] OR dogs[tiab] OR "canis familiaris"[tiab] OR "Cats"[Mesh] OR (cat[tiab] AND (animal[tiab] OR animals [Mesh:noexp] OR domestic*[tiab])) OR cats[tiab] OR "felis catus"[tiab] OR "Mink"[Mesh] OR mink[tiab] OR minks[tiab] OR mustela[tiab] OR "Rabbits"[Mesh] OR (rabbit*[tiab] AND domestic*[tiab]) OR "Oryctolagus cuniculus"[tiab] OR "Cricetinae"[Mesh] OR hamster*[tiab]	
#5	Search #3 OR #4	65,913
#4	Search "Nitrates/adverse effects"[Mesh] OR "Nitrates/toxicity"[Mesh] OR "Nitrates/ poisoning"[Mesh] OR "Nitrates/pharmacokinetics"[Mesh] OR "Nitrites/adverse effects"[Mesh] OR "Nitrites/pharmacokinetics"[Mesh] OR "Nitrites/poisoning"[Mesh] OR "Nitrites/toxicity"[Mesh]	4,004
#3	Search #1 AND #2	64,741
#2	Search "Long Term Adverse Effects"[Mesh] OR effect[tiab] OR effects[tiab] OR tolerabilit*[tiab] OR safe[tiab] OR safety[tiab] OR "Toxicology"[Mesh] OR toxico* [tiab] OR toxici*[tiab] OR cardiotox*[tiab] OR genotox*[tiab] OR hepatotox*[tiab] OR immunotox*[tiab] OR nephrotox*[tiab] OR neurotox*[tiab] OR "Toxicity Tests"[Mesh] OR mutagen*[tiab] OR terato*[tiab] OR terata*[tiab] OR lethal dos* [tiab] OR LD50[tiab] OR NOAEL[tiab] OR "Toxic Actions"[Mesh:noexp] OR "Sheep Diseases"[Mesh] OR "Animal Diseases"[Mesh:NoExp] OR "Cattle Diseases"[Mesh] OR "cat diseases"[Mesh] OR "dog diseases"[Mesh] OR "horse diseases"[Mesh] OR "sheep diseases"[Mesh] OR "swine diseases"[Mesh] OR "poultry diseases"[Mesh] OR disease*[tiab] OR illness*[tiab] OR carcinogen*[tiab] OR cancer [sb] OR "Methemoglobinemia"[Mesh] OR methemoglob*[tiab] OR methaemoglob*[tiab] OR "Hyperplasia"[Mesh] OR hyperplas*[tiab] OR "Pharmacokinetics"[Mesh] OR Pharmacokinetic*[tiab] OR pharmacodynamic*[tiab] OR "Toxicokinetics"[Mesh] OR administration[tiab] OR absorption[tiab] OR distribution[tiab] OR resorption[tiab] OR bioavailab*[tiab] OR metaboli*[tiab] OR biotransform*[tiab] OR activat*[tiab] OR half-li*[tiab] OR excret*[tiab] OR clearance[tiab] OR eliminat*[tiab] OR bioconcentrat*[tiab] OR PBPK[tiab] OR PBK[tiab] OR ADME[tiab] OR "Hematology"[Mesh:NoExp] OR hematolog*[tiab] OR haematolog*[tiab] OR "Abortion, Veterinary"[Mesh] OR abortion*[tiab] OR "Reproductive Physiological Phenomena"[Mesh] OR reproduction[tiab] OR testicul*[tiab] OR sperm[tiab] OR "Sperm Count"[Mesh] OR "Epididymitis"[Mesh] OR epidid*[tiab] OR "Cardiovascular Diseases"[Mesh] OR cardiovasc*[tiab] OR tachycardi*[tiab] OR hypertens*[tiab] OR "Hypoxia"[Mesh] OR hypox*[tiab] OR diabet*[tiab] OR "Gastrointestinal Tract"[Mesh] OR "gastrointestinal tract"[tiab] OR "GI tract"[tiab] OR gastric[tiab] OR digestive[tiab] OR intestin*[tiab] OR "Saliva"[Mesh] OR saliva[tiab] OR "Adenoma"[Mesh] OR adenoma*[tiab] OR "Stomach, Ruminant"[Mesh] OR rumen*[tiab] OR forestomach* [tiab] OR "Oropharynx"[Mesh] OR oropharyn*[tiab] OR "Muscle Weakness"[Mesh] OR weakness[tiab] OR "Vasodilator Agents"[Mesh] OR vasodilator*[tiab] OR "Liver"[Mesh] OR liver[tiab] OR "Urine"[Mesh] OR "Polyuria"[Mesh] OR polyuri*[tiab] OR "Thyroid Gland"[Mesh] OR thyroid*[tiab]	18,040,976
#1	Search "Nitrates"[Mesh:NoExp] OR "Nitrites"[Mesh:NoExp] OR "Sodium Nitrite"[Mesh] OR nitrate*[tiab] OR nitrite*[tiab] OR 7631-99-4[tiab] OR 7757-79-1[tiab] OR 6484-52-2 [tiab] OR "7632-00-0"[tiab] OR 7631994[tiab] OR 7757791[tiab] OR 6484522 [tiab] OR 7632000[tiab]	95,674

Web of Science Platform

Date of the search: 13/02/2020

Set	Query	Results
# 9	(#8) *AND* LANGUAGE: (English) *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC*	3,725
# 8	#6 NOT #7 *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	3,926
# 7	(TS=(salami OR sausage* OR curing OR cured OR ham OR jambon OR cytometry OR manure OR leach* OR sludge OR "PROCESSED MEAT" OR "red meat" OR "self life" OR rat OR rats OR mice OR mouse OR monkey OR monkeys OR zebrafish OR medaka OR "human milk")) OR (TI=(human OR humans)) *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	4,706,4286
# 6	#5 AND #2 AND #1 *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	6,473
# 5	#4 OR #3 *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	17,452,112
# 4	TS=(((adverse OR undesirable OR harm* OR serious) NEAR/5 (outcome* OR effect OR effects)) OR tolerabilit* OR safe OR safety OR toxico* OR toxici* OR cardiotox* OR genotox* OR hepatotox* OR immunotox* OR nephrotox* OR neurotox* OR mutagen* OR terato* OR terata* OR "lethal dos*" OR LD50 OR NOAEL OR disease* OR illness* OR carcinogen* OR cancer* OR tumor* OR tumour* OR neoplasm* OR methemoglob* OR methaemoglob* OR hyperplas* OR Pharmacokinetic* OR pharmacodynamic* OR administration OR absorption OR distribution OR bioavailab* OR metaboli* OR biotransform* OR activat* OR "half li*" OR excret* OR clearance OR eliminat* OR bioconcentrat* OR PBPK OR PBK OR ADME OR hematolog* OR haematolog* OR abortion* OR reproduction OR testicul* OR sperm OR epidid*OR cardiovasc* OR tachycardi* OR hypertens* OR hypox* OR diabet* OR "gastrointestinal tract" OR "GI tract" OR gastric OR digestive OR intestin* OR saliva OR adenoma* OR rumen* OR forestomach OR oropharyn* OR weakness OR vasodilator* OR liver OR polyuri* OR urine OR thyroid*) *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	15,543,024
# 3	TI=(effect OR effects) *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	2,977,454
# 2	TS=(nitrate* OR nitrite* OR 7631-99-4 OR 7757-79-1 OR 6484-52-2 OR "7632-00-0" OR 7631994 OR 7757791 OR 6484522 OR 7632000) *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	211,011
# 1	TS=((((domestic OR farm) NEAR/5 animal*) OR ruminat* OR cattle OR cow OR cows OR bull OR bulls OR calf OR calves OR heifer* OR bullock* OR veal OR veal* OR "dairy herd*" OR "dairy breed*" OR bovine* OR ovis OR ovine OR ewe OR ewes OR lamb OR lambs OR sheep* OR goat OR goats OR capra OR capras OR caprin* OR buffalo* OR bubalus OR swine OR "sus scrofa " OR "sus domestica" OR "sus domesticus" OR porcine OR suidae OR pig OR pigs OR piglet* OR sow OR sows OR barrow* OR boar OR boars OR hog OR hogs OR gilt OR gilts OR poultry OR Poultries OR (domestic* NEAR/3 (bird OR birds)) OR fowl OR fowls OR galliform* OR wildfowl* OR "gallinaceous bird" OR landfowl OR chicken* OR "Gallus gallus" OR "Gallus domesticus" OR broiler* OR capon OR capons OR cockerel* OR hen OR hens OR pullet OR pullets OR rooster OR roosters OR waterfowl* OR Anatidae OR duck OR ducks OR mallard* OR "Anas platyrhynchos" OR Geese OR goose OR anser OR branta OR Coturnix OR quail* OR (turkey AND (animal* OR bird OR birds)) OR Turkeys OR meleagris OR pigeon* OR dove OR doves OR columb* OR ostrich OR ostriches OR equidae* OR equus OR horse* OR	2,184,556

Set	Query	Results
	equine* OR colt OR colts OR foal OR foals OR yearling* OR gelding* OR mare OR mares OR pony OR ponies OR stallion* OR filly OR fillies OR ass OR asses OR mule OR mules OR donkey* OR pets OR pet OR dog OR dogs OR "canis familiaris" OR (cat AND (animal OR domestic*)) OR cats OR "felis catus" OR mink OR minks OR mustela OR (rabbit* AND domestic*) OR "Oryctolagus cuniculus" OR hamster*)) *Indexes=SCI-EXPANDED, ESCI, CCR-EXPANDED, IC Timespan=All years*	

Table I.1: Overview of retrieval of references concerning adverse effects of nitrate and nitrite in feed

Database	Search Adverse Effects
PubMed	3,914
Web of Science	3,725
Searches merged, after de-duplication	6,449

Table I.1 shows that duplicates were removed after merging retrieved references in individual searches. The duplicates were removed through both Endnote and Distiller software.

The references were subsequently screened for relevance and characterised in relation to the areas of interest based on their titles and abstracts.

The studies were excluded according to the following criteria:

- Positive effects of nitrate and nitrite
- Effects in fish from nitrate and nitrite in water
- Nitrates and nitrite in the environment
- Reviews or abstract only
- Date before 1950
- Abstract not in English

Screening for relevance and study characterisation

The titles and the abstracts of the articles retrieved were screened for their potential relevance by two reviewers and were separated according to species.

The abstracts proposed as potentially relevant were then screened by the working group (WG) members and, by applying expert judgement were used in the assessment if considered relevant for animal risk assessment. The previous assessment of EFSA (EFSA, 2009) was also considered for the present assessment. Whenever necessary, original publications referenced in this previous assessment were retrieved and re-assessed.

In addition to the systematic search and the use of the previous evaluation for retrieval of relevant literature, a 'forward snowballing' approach was applied by all WG members in order to obtain any relevant information published.

Results

A total of 6,449 references were screened for relevance based on their titles and abstracts. Out of these, 317 references were selected via Distiller as relevant to be further screened by the Working Group experts.

Annex II – Animal example diets

Methodology for estimating exposure by farm and companion animals to nitrate

This Annex gives details of animal live weights, productivity and feed and water intakes for farmed livestock and companion animals used to estimate exposure to nitrate in this Opinion. In the absence of a standard database of feed intakes for the EU livestock, published guidelines on nutrition and feeding (AFRC, 1993; Carabaño and Piquer, 1998; NRC, 2007a,b; Leeson and Summers, 2008; EFSA FEEDAP Panel, 2012; OECD, 2013; McDonald et al., 2011) have been used to formulate diets. Therefore, the estimates of exposure are in agreement with common practice but do not represent worst-case scenarios.

Based on these estimates of intake, the mean lower bound (LB) and upper bound (UB) concentrations of nitrate in the estimated diets for the farm livestock species and companion animals have been calculated, and are reported in Table 10 of this Opinion.

Feeds and feed intakes

Cattle, sheep, goats and horses.

The diets of cattle, sheep, goats and horses consist predominantly of forages supplemented mainly with cereal grains, vegetable proteins and by-products of food production. Forages may be fed fresh or conserved (e.g. as hay or silage). In this Opinion, estimates of exposure are given for ruminant livestock fed grass silage or maize silage-based diets. Horses, sheep and goats are not normally fed on maize silage, and therefore, estimates are only given for grass silage-based diets.

Table II.1: Live weights, growth rate/productivity, dry matter intake for cattle, sheep, goats and horses, and the proportions of the diet as non-forage

Animal species	Live weight (kg)	Growth rate or productivity	Dry matter intake (kg/day)	% of diet as non-forage feed	Reference
Dairy cows, lactating[(a)]	650	40 kg milk/day	20.7	40	AFRC (1993)
Fattening cattle: beef[(b)]	400	1 kg/day	9.6	15	AFRC (1993)
Fattening cattle: maize silage-based ration	300	1.4 kg/day	6.6	25	Browne et al. (2004)
Sheep: lactating	80	Feeding twin lambs	2.8	50	AFRC (1993)
Goats: milking[(a)]	60	6 kg milk/day	3.4	65	NRC (2007a)
Goats: fattening	40	0.2 kg/day	1.5	40	NRC (2007a)
Horses	450	Moderate activity	9.0	50	NRC (2007b)

(a): Months 2–3 of lactation. Grass silage-based diet.
(b): Housed castrate cattle, medium maturing breed.

Non-ruminant animals

Pigs

Although there is a considerable range of pig production systems in Europe, exposure estimates have been made for piglets (pig starter), finishing pigs and lactating sows, using feed intakes proposed by EFSA FEEDAP Panel (2012). Details are given in Annex Table II.2.

Poultry

The CONTAM Panel applied the live weights and feed intakes reported for fattening chickens (broilers), laying hens and turkeys proposed by EFSA FEEDAP Panel (2012) and for ducks by Leeson and Summers (2008) (see Annex Table II.2).

Rabbits

Feed intakes of 65–80 g/kg bw per day have been reported (Carabano and Piquer, 1998). For the exposure estimates, the CONTAM Panel have assumed a live weight of 2 kg, and a daily feed intake of 75 g/kg bw (derived from Carabano and Piquer, 1998).

Table II.2: Live weights and feed intake for pigs, poultry (EFSA FEEDAP Panel, 2012), ducks (Leeson and Summers, 2008) and rabbits

Animal species	Live weight (kg)	Feed intake (kg dry matter/day)	Reference
Pigs: starter	20	1.0	EFSA FEEDAP Panel (2012)
Pigs: finishing	100	3.0	EFSA FEEDAP Panel (2012)
Pigs: lactating sows	200	6.0	EFSA FEEDAP Panel (2012)
Poultry: broilers[(a)]	2	0.12	EFSA FEEDAP Panel (2012)
Poultry: laying hens	2	0.12	EFSA FEEDAP Panel (2012)
Turkeys: fattening	12	0.40	EFSA FEEDAP Panel (2012)
Ducks: fattening	3	0.14	Leeson and Summers (2008)
Rabbits	2	75	Carabaño and Piquer (1998)

(a): Fattening chickens.

Companion animals: Dogs and cats

The amount of food consumed is largely a function of the mature weight of the animal, level of activity, physiological status (e.g. pregnancy or lactation) and the energy content of the diet. In this Scientific Opinion, the CONTAM Panel assumed body weights (kg) and feed intakes (g dry matter/day) for dogs and cats of 25/360 and 4/60, respectively (derived from NRC, 2006).

B.2. Diet composition

In the absence of any data on levels of nitrate in compound feeds, estimates of exposure have been made using dietary inclusion rates of individual feed materials *for which data on levels of nitrate were available*. It should be noted that for many feed materials commonly used in diets of farmed and companion animals, no data on levels of nitrate were available, and therefore, in the diets reported below, the sums of the ingredients do reflect the whole diet and therefore do not equal 100%.

Cattle, sheep, goats and horses

Diets of ruminants and horses consist of forages (either fresh or conserved as silage or hay) supplemented with non-forage feeds such as cereals, cereal by-products, oilseed meals and by-products of human food production. Annex Table II.3 provides details of the proportions of concentrate (non-forage) feeds in the diets of ruminants which, in the case of dairy cows, is assumed which total to 40%. The remaining 60% is assumed to be the percentage of forage feeds.

Table II.3: Assumed levels of feeds in diets (%) for ruminants and horses fed grass silage-based diets

Livestock category		Dairy: high yielding	Beef: fattening	Sheep: lactating	Goats: lactating	Goats: fattening	Horses
% of non-forage feeds in the total diet	**Wheat**	**6**		**7**			
	Barley	**8**	**6**	**9**	**37.5**	**16**	
	Oats						**6.4**
	RSM	**8**	**3**	**5**	**7.5**	**4**	
	Sunflower meal			**2.5**			
	Maize gluten feed	**4**	**1.65**		**7.5**	**8**	
	Wheat feed	**4**	**1.5**	**7.5**	**7.5**	**4**	**5.6**
	Lucerne meal						**2**
	Other feeds*	**10**	**2.85**	**19**	**15**	**8**	**6**
% of forage feed in the total diet		**60**	**85**	**50**	**75**	**60**	**80**

ni: Not included in the diet formulations.
*: Includes feeds for which no data were available or in which nitrates would not be expected, e.g. mineral supplements.

For maize silage-based diets, exposures have been estimated for lactating dairy cows and fattening beef cattle fed diets described in Annex Table II.4.

Table II.4: Maize silage-based diets for dairy cows and beef cattle

Animal species	Quantities of feed consumed (kg dry matter/day)					Reference
	Forage	Maize grain	Soybean meal	Barley grain	Rapeseed meal	
Lactating dairy cows: maize silage-based diet	15.0	9.5	2.8	ni	ni	AFSSA (2009)
Fattening beef cattle: maize silage-based diet	4.9	ni	ni	ni	1.5	EBLEX (2012)

ni: not included in the diet formulations.

Pigs and poultry

Since no data on levels of nitrate in species-specific compound feeds for pigs or poultry were available, example diets were formulated intended to reflect current feeding systems. Details are given in Annex Table II.5.

Rabbits

In this opinion, the feed ingredients used in a typical French commercial rabbit compound, as provided by T. Gidenne (Personal communication, 2011), have been used, details of which are given in Annex Table II.5.

Companion animals (dogs and cats)

The European Pet Food Industry Federation (FEDIAF) has provided information on typical inclusion levels of cereals, cereal by-products and other feed materials in dry cat and dog food.[31] In the absence of sufficient data on species-specific manufactured complete feedingstuffs, the CONTAM Panel has used example diets based on this information in Annex Tables II.5 and II.6.

Table II.5: Assumed diet composition (%) for pigs and poultry

Feed materials	Pigs			Chickens			Turkeys	Ducks
	Starter	Finisher	Lactating sow	Broilers: starter	Broilers: growers	Laying hens	Growers	Growers
Wheat	48	48	50	32	38	30	30	45
Barley	16	20	11	ni	ni	ni	35	15
Maize	ni	ni	ni	35	38	35	ni	ni
SBM	20	11	16	25	15	22	15	28
RSM	2.5	4	ni	ni	ni	ni	ni	ni
Lucerne meal	ni	ni	ni	ni	ni	4	9	5
Wheatfeed	3	8	14		1	1	3	2
Molasses	3	4	4	3	3	3	3	ni

ni: Not included in the diet formulations.

[31] The European Pet Food Industry Federation (FEDIAF), Personal communication by email, May 2016.

Table II.6: Assumed diet composition (%) for farmed rabbits and companion animals (cats and dogs)

Feed materials	Farmed rabbits	Companion animals	
		Cats	Dogs
Wheat (%)	ni	10	10
Barley (%)	ni	ni	ni
Maize (%)	17.6	5	6
Oats (%)	ni	1	0.5
Rapeseed meal (%)	ni	ni	ni
Maize gluten meal (%)	ni	17	15
Sunflower meal (%)	20.0	ni	ni
Lucerne meal (%)	19.1	ni	ni
Wheat feed (%)	18.3	12	20
Meat meal (%)	ni	38	40

ni: not included in the diet formulations.

Exposure of livestock and companion animals to nitrate from water consumed

Within species, water consumption is influenced by many factors but principally by ambient temperature, diet composition and particularly moisture content), physiological state and level of activity and productivity. Data for livestock have been published by a number of national authorities and summarised in OMAFRA (2007). Mean LB and UB nitrate concentrations in tap water of 14.8 (LB) and 14.9 (UB) mg/L were retrieved from the EFSA database on chemical occurrence data. Estimates of intake of nitrate from feed, water, as well as from feed and water combined, are given in Table II.7.

Table II.7: Water intake and its contribution to total nitrate exposure by livestock and companion animals

Animal species (and diet)		Body weight (kg)	Water intake L/day	Nitrate intake from water (mg/day)	Nitrate intake from feed (mg/d)	Total intake from feed+water (mg/day)	Intake from feed+water, (mg/kg bw per day)
Dairy cows: Grass silage	LB	650	115	1,781	32,673	34,454	53
	UB	650	115	1,793	32,680	34,473	53
Dairy Cows: Maize silage	LB	650	115	1,781	14,683	16,464	25
	UB	650	115	1,793	14,701	16,494	25
Beef cattle: Grass silage	LB	400	41	445	21,323	21,768	54
	UB	400	41	448	21,324	21,772	54
Beef cattle: maize silage	LB	300	41	448	4,386	4,835	16
	UB	300	41	448	4,389	4,837	16
Sheep: lactating	LB	80	10	104	3,703	3,807	48
	UB	80	10	105	3,704	3,809	48
Goats: lactating	LB	60	15	223	2,422	2,645	44
	UB	60	15	224	2,424	2,648	44
Goats: fattening	LB	40	6	89.0	2,383	2,472	62
	UB	40	6	89.6	2,384	2,473	62
Horses	LB	450	45	667	5,790	6,458	14
	UB	450	45	672	5,795	6,467	14
Pig starter	LB	20	2	29.7	39.6	69.3	3.5
	UB	20	2	29.9	40.6	70.5	3.5
Pig finisher	LB	100	9	148	109	258	2.4
	UB	100	9	149	112	262	2.5
Pig: lactating sow	LB	200	20	371	141	512	2.2

Animal species (and diet)		Body weight (kg)	Water intake L/day	Nitrate intake from water (mg/day)	Nitrate intake from feed (mg/d)	Total intake from feed+water (mg/day)	Intake from feed+water, (mg/kg bw per day)
	UB	200	20	374	147	520	2.2
Chickens: fattening	LB	2.0	0.47	2.23	6.79	9.01	6.9
	UB	2.0	0.47	2.24	6.93	9.17	6.9
Chickens: laying hens	LB	2.0	0.32	1.71	11.0	12.7	7.9
	UB	2.0	0.32	1.72	11.1	12.9	8.0
Turkeys: fattening	LB	12	0.80	10.4	57.3	67.7	5.8
	UB	12	0.80	10.5	57.6	68.1	5.8
Ducks: fattening	LB	3.0	1.20	17.8	10.5	28.3	9.5
	UB	3.0	1.20	17.9	10.6	28.6	9.5
Cats	LB	4.0	0.20	2.97	1.02	3.99	1.0
	UB	4.0	0.20	2.99	1.05	4.04	1.0
Dogs	LB	25	1.40	20.8	8.96	29.7	1.2
	UB	25	1.40	20.9	9.17	30.1	1.2
Rabbits	LB	2.0	1.02	2.97	4.98	8.0	10
	UB	2.0	1.02	2.99	5.06	8.0	10

LB: lower bound; UB: upper bound.

Annex III – Occurrence data

Annex III together with the corresponding cvs file including the raw data can be found as separate documents available online on the EFSA Knowledge Junction community at: https://doi.org/10.5281/zenodo.4061688

Description: This Annex is an Excel file which presents tables on occurrence data of nitrate and nitrite in Feed.

Annex IV – EFSA guidance documents applied for the risk assessment

- EFSA (European Food Safety Authority), 2006. Guidance of the Scientific Committee on a request from EFSA related to uncertainties in Dietary Exposure Assessment. EFSA Journal 2006;4(5):438, 54 pp. https://doi.org/10.2903/j.efsa.2006.438
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