SCIENTIFIC OPINION

Nitrite as undesirable substances in animal feed

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question N° EFSA-Q-2005-287)

Adopted on 25th March 2009

PANEL MEMBERS


SUMMARY

Nitrite is formed naturally by the nitrogen cycle during the process of nitrogen fixation and it is subsequently converted to nitrate, a major nutrient assimilated by plants. Two main nitrite salt forms occur, namely sodium and potassium nitrite.

In animal tissues, nitrite is naturally present mainly as the result of endogenous nitrate conversion. Animal feed represents a natural source of exogenous nitrite and because of its potential for toxicity at excessive levels of intake, the Directive (EC) No 2002/32/EC on undesirable substances in animal feed established maximum limits for sodium nitrite in complete feedingstuffs excluding feedingstuffs intended for pets except birds and aquarium fish, and fish meal of 15 and 60 mg/kg, respectively (corresponding to 10 and 40 mg/kg for the nitrite ion). Nitrite in drinking water is regulated in Europe, with a maximum level of 0.5 mg/L. Nitrate levels in forages are naturally high and the inter-conversion of nitrate to nitrite is the largest contributor to nitrite exposure in food-producing animals. Dietary intake of nitrite per se results from the use of vegetable feeds and forages, fertilisers/manures or when used as an antimicrobial for preservation, for example in silage, processed pet food, and in former times fish meal. Additionally, water can be an important dietary source of nitrite via reduction of nitrate. The main analytical technique used to measure both nitrite and nitrate is colourimetry because of its sensitivity and specificity. Three member states provided analytical results on feedingstuffs and nitrite levels were below the maximum levels in all feed commodities.

Acute toxicity, nitrite is approximately ten-fold more toxic than nitrate and three main toxicological endpoints have been identified: methaemoglobin formation (in a wide range of species including man), hypertrophy of the adrenal zona glomerulosa (rats), and equivocal evidence

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The Acceptor Daily Intake (ADI) for nitrite 0-0.07 mg/kg body weight (b.w.) per day has been endorsed by the Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority during the recent risk benefit assessment of nitrate in vegetables.

In monogastric animals, most nitrite is formed and absorbed in the upper digestive tract. In contrast in ruminants, nitrite and nitrate is metabolised by the rumen flora. Reports of adverse effects after excessive nitrite exposure in livestock exist, and pigs and ruminants, as major food producing animals, are particularly susceptible: this is because of relatively low nitrite reductase activity and high levels of rumen conversion of exogenous nitrate to nitrite, respectively. Acknowledging diverse husbandry conditions between reported studies, No-Observed Adverse Effect Levels (NOAELs) for pigs and cattle have been estimated from the literature with a value of 3.3 mg/kg b.w. per day in both species. In turn, total daily nitrite intakes were estimated for pigs and cattle using the maximum exposure level in complete feed according to the current legislation (10 mg/kg), typical feeding regimens within the European Union and maximum nitrite level in forages from member states (cattle). Overall, the estimated nitrite intakes for pigs and cattle from feed were 0.37 and 0.65 mg/kg b.w. per day (excluding endogenous formation of nitrite) respectively, corresponding to margins of safety of 9 and 5 in comparison with the respective NOAELs. The CONTAM Panel considered that such levels do not pose concerns for animal health given that livestock are husbanded under good agricultural practices. The protection of livestock health is further reinforced by the awareness of livestock producers, of the conditions that are likely to lead to nitrite poisoning particularly regarding high levels of nitrate in forages and the inter-conversion of nitrate to nitrite.

The CONTAM Panel concluded that the typical daily human dietary exposure to nitrite from fresh animal products (e.g. milk, meat and eggs) is only (2.9 %) of the total daily dietary exposure to nitrite. The CONTAM Panel concludes that such low nitrite levels in fresh animal products do not raise any concern for human health.

Key words: nitrite, feedingstuffs, toxicity, exposure, carry-over, animal health, human health.
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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

1. General background


The main modifications can be summarised as follows:

- extension of the scope of the Directive to include the possibility of establishing maximum limits for undesirable substances in feed additives;
- deletion of the existing possibility to dilute contaminated feed materials instead of decontamination or destruction (introduction of the principle of non-dilution);
- deletion of the possibility for derogation of the maximum limits for particular local reasons;
- introduction the possibility of the establishment of an action threshold triggering an investigation to identify the source of contamination (“early warning system”) and to take measures to reduce or eliminate the contamination (“pro-active approach”).

In particular the introduction of the principle of non-dilution is an important and far-reaching measure. In order to protect public and animal health, it is important that the overall contamination of the food and feed chain is reduced to a level as low as reasonably achievable providing a high level of public health and animal health protection. The deletion of the possibility of dilution is a powerful means to stimulate all operators throughout the chain to apply the necessary prevention measures to avoid contamination as much as possible. The prohibition of dilution accompanied with the necessary control measures will effectively contribute to safer feed.

During the discussions in view of the adoption of Directive 2002/32/EC the Commission made the commitment to review the provisions laid down in Annex I on the basis of updated scientific risk assessments and taking into account the prohibition of any dilution of contaminated non-complying products intended for animal feed. The Commission has therefore requested the Scientific Committee on Animal Nutrition (SCAN) in March 2001 to provide these updated scientific risk assessments in order to enable the Commission to finalise this review as soon as possible (Question 121 on undesirable substances in feed)\(^4\).

The opinion on undesirable substances in feed, adopted by SCAN on 20 February 2003 and updated on 25 April 2003\(^5\) provides a comprehensive overview on the possible risks for animal and public health as the consequence of the presence of undesirable substances in animal feed.

It was nevertheless acknowledged by SCAN itself and by the Standing Committee on the Food Chain and Animal Health that for several undesirable substances additional detailed risk assessments are necessary to enable a complete review of the provisions in the Annex.

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\(^3\) OJ L 115, 4.5.1999, p. 32.
2. Specific background

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 nitrates. Conversely, microbiological conversion of nitrate to nitrite may also occur, for instance in the digestive tract.

Nitrite is widely used in the processing and preservation of certain meat products. Nitrite has been used in some countries for the preservation of fish meal submitted to heat treatment, but is no longer permitted as it has been suspected to generate nitrosamines when reacting with higher amines present in fish.


SCAN concluded that the ions and elements, including nitrite, listed in Council Directive 2002/32/EC are commonly encountered substances with known toxicity. In each case, the contribution of food products of animal origin to the human exposure is limited and listing of these elements as undesirable substance in feed, although concomitantly contributing to an overall reduction of human exposure to toxic forms, is mainly justified by reasons of animal health.

SCAN concluded furthermore that a detailed risk assessment of the presence of nitrite in animal feed and the possible effects for animal health and public health is necessary. Nitrite is an endogenous compound naturally present in feed materials of plant and animal origin. The natural levels in commercially available feedstuffs have not been reported to cause intoxication of farm animals. As a consequence, retaining a maximum level for nitrite in feedstuffs appears to serve no practical purpose. Therefore, SCAN is of the opinion that nitrite could be removed from the Annex to the Directive 2002/32/EC. The requested detailed assessment should provide information to judge if this conclusion from SCAN can be confirmed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) of Regulation (EC) No 178/2002 the European Commission asks the European Food Safety Authority requests to provide a scientific opinion on the presence of nitrite in animal feed.

This detailed scientific opinion should comprise the

• determination of the toxic exposure levels (daily exposure) of nitrite for the different animal species (difference in sensitivity between animal species) above which
  • signs of toxicity can be observed (animal health / impact on animal health) or
  • the level of transfer/carry over of nitrite from the feed to the products of animal origin results in unacceptable levels of nitrite in the products of animal origin in view of providing a high level of public health protection.

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6 Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 6.11. Conclusions and recommendations.
identification of feed materials which could be considered as sources of contamination by nitrite and the characterisation, insofar as possible, of the distribution of levels of contamination

assessment of the contribution of the different identified feed materials as sources of contamination by nitrite
  - to the overall exposure of the different relevant animal species to nitrite
  - to the impact on animal health,
  - to the contamination of food of animal origin (the impact on public health), taking into account the dietary variations and variable carry over rates (bio-availability) depending on the nature of the different feed materials.

identification of eventual gaps in the available data which need to be filled in order to complete the evaluation.

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Nitrite as undesirable substances in animal feed

ASSESSMENT

1. Introduction

Nitrite is the anion of inorganic nitrite salts, the most important of which are sodium nitrite and potassium nitrite. It is formed naturally by the nitrogen cycle during the process of nitrogen fixation. It is subsequently converted to nitrate, a major plant nutrient. In vertebrates, nitrate is converted to nitrite and other metabolites (nitric oxide and N-nitroso compounds) either in the saliva of most monogastric animals or in the stomach of ruminants due to microbiological action. Exposure to nitrite has been associated with potential adverse health implications, acutely methaemoglobin formation and chronically gastric neoplasia in mice, for which in the case of the latter clear evidence in man is not confirmed. Reports of adverse effects in livestock exist due to nitrite feed contamination and pigs and ruminants are particularly susceptible to its toxicity, based on their respective physiology. Overall, the acute toxicity of nitrite has been shown to be approximately 10-fold higher than that of nitrate, and because of its natural presence in plants, silages, forages, water and feed, the Directive 2002/32/EC limits its content in commercial feed used for livestock, fish and pets, excluding birds and aquarium fish.

The first international evaluation of the risks associated with the ingestion of nitrite was conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1961 (FAO/WHO, 1962). The Scientific Committee for Food (SCF) reviewed the toxicological effects of nitrite and established temporary Acceptable Daily Intakes (ADI) of 0-0.1 mg/kg b.w. per day in 1990 (EU, 1992) and 0-0.06 mg/kg b.w. per day in 1995 (EU, 1995). In 2002, the JECFA set an ADI of 0-0.07 mg/kg b.w. per day (FAO/WHO, 2003a,b). An Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/kg b.w. per day, was established by the former SCF and was reconfirmed by the JECFA in 2002 and by the Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA) in 2008 (EFSA, 2008a).

In order to perform a risk assessment for nitrite in feed for animals and human health, the potential sources of nitrite exposure have been identified as feed and drinking water for animal health and animal products for human health. However, there are multiple other sources and salts of nitrite (plants, soil, standing water, as well as inter-conversion from nitrate from feed, plants and water). In consequence because of the difficulty of estimating the overall nitrite exposure for each livestock species under the full range of typical husbandry situations, the present opinion focuses on the impact of nitrite in feed on animal and human health. For convention, the term nitrite, which has been used throughout this report, refers to the ion (NO$_2^-$).

1.1. Chemistry, production and use of nitrite

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 nitrates. 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.

Nitrite (NO$_2^-$) is the anion of inorganic nitrite salts, the most important of which are sodium nitrite and potassium nitrite.
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Sodium nitrite, with the chemical formula NaNO₂, is a white to slightly yellowish crystalline powder. It has a solubility of 82 g/100 mL water at 20°C. It is hygroscopic. It has a melting point of 270°C and decomposes above 320°C. It slowly oxidizes in the air to sodium nitrate.

It is used as an antimicrobial agent, a preservative and a colour fixative in meats and fish. In the European Union (EU) and, when labelled ‘for food use’, sodium nitrite may only be sold in a mixture with salt (NaCl) or a salt substitute. Sodium nitrite is known as E250. As a food additive it stabilizes the colour of preserved fish and meats and also it inhibits the growth of Clostridium botulinum, the bacterium, which produces the botulinum toxin. Maximal residual amounts, expressed as mg NaNO₂/kg have been set for various types of meat products (Part C of Annex III of Council Directive N° 95/2/EC). It is also used in the manufacturing of azo dyes, nitroso compounds and other organic compounds. Other applications are: dyeing and printing of textile fabrics, bleaching of fibres, in photography, in metal coatings for phosphatising and detinning and as an analytical reagent.

It has been used in human and veterinary medicine, as a vasodilator, a 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. The residues of nitrite in milk from cows treated with sodium nitrite have been shown to be below the limit of detection so that such residues in milk can be considered negligible (EMEA, 2006). The Committee for Medicinal Products for veterinary use (CVMP), recommended in 2006 the inclusion of sodium nitrite for bovine species for topical use only in Annex II of Council Regulation (EEC) n° 2377/90.

Potassium nitrite, with chemical formula KNO₂, is a white to slightly yellow crystalline powder. The melting point is at 441°C but decomposition starts at 350°C. It is very soluble in water. After drying it contains not less than 95 % KNO₂, the remainder consisting chiefly of nitrate. 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 nitrates. Like sodium nitrite it inhibits the growth of the botulism-causing bacterium Clostridium botulinum. As a permitted food additive, it is known in the EU as E249.

1.2. Hazard assessment for humans

Dietary studies from the UK (MAFF, 1998) and France (Causeret, 1984) were used by EFSA (EFSA, 2008a) to estimate the average exogenous intake of nitrite (1.5-2.0 mg/person b.w. per day) in humans. From these total dietary estimates, the major contributors to exogenous dietary nitrite are vegetables and fruit (11-41 %), animal-based products (35-39 %), of which non-preserved animal products (milk, dairy products fresh meat, fish and eggs) represent 14 %, water (5-7 %) and other food (15-47 %), approximating to 1.5 to 2.0 mg/person/day. In quantitative terms, dietary nitrite represents less than 20 % (15-17 %) of the total daily nitrite exposure, (7.3 to 11.3 mg/person/day), the remaining 80 % (83-85 %) resulting from the endogenous bioconversion of dietary nitrate to nitrite in saliva; key sources of nitrate being vegetables and fruit (52-75 %) and water (14-22 %) (EFSA, 2008a).

Historically, risk assessments for humans from nitrate and nitrite dietary exposure have been performed by the SCF and JECFA (EC, 1992, 1995; FAO/WHO, 2003a,b) and more recently by the CONTAM Panel of EFSA as a risk benefit assessment of nitrate in vegetables. Overall, there were no new toxicological data available that would impact the Acceptable Daily

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Intakes (ADI) for either nitrate or nitrite so the JECFA values were kept in the CONTAM Panel's assessment (EFSA, 2008a).

The following section presents a short summary of the toxicology of nitrite with particular emphasis on the potential for human health effects. Nitrate is not discussed in detail as it is not the subject of the terms of reference, although the critical studies leading to its ADI are presented for completeness as these illustrate its comparative toxicity in relation to nitrite.

The acute toxicity of sodium nitrite is approximately 10-fold higher than that of nitrate with LD₅₀ values of 214, 180, 186 mg/kg b.w. in mice, rats and in rabbits (corresponding to 143, 121, 126 mg/kg b.w. for nitrite, respectively) compared to 2500-6250, 3300-9000, 1900-2680 mg/kg b.w. for sodium nitrate (corresponding to 1525-3810, 2440-6660, 1410-2000 for mg/kg b.w. for nitrate) (NIOSH, 1987; Speijers, 1987; Walker, 1990).

A 14 week drinking water study was conducted in B6C3F1 mice (10 males and 10 females/group) at dose levels ranging from 90-1230 mg/kg b.w. per day sodium nitrite (corresponding to 60-824 mg/kg b.w. per day for nitrite). Overall at the highest dose, body weight, spleen weight and sperm counts were lower in males compared to controls. In females, absolute and relative organ weights (heart, kidney, liver and spleen), and the length of oestrous cycle were impaired. Squamous cell hyperplasia of the forestomach and extramedullary hematopoiesis were more frequent at the two highest nitrite levels in both sexes as well as degeneration of testis in males resulting in a NOAEL of 190 mg/kg b.w./day (corresponding to 127 mg/kg b.w. per day for nitrite) for this species (National Toxicology Programme NTP, 2001). A 14 week drinking water study was conducted in male and female rats (10 males and 10 females per group) at dose levels of 30-340 mg/kg b.w. per day of sodium nitrite (corresponding to 20-228 mg/kg b.w. per day for nitrite). Elevated methaemoglobin was observed at all doses. Sperm motility was used as the endpoint to determine a no observed effect level (NOEL) of 55 mg/kg b.w. per day for sodium nitrite corresponding to 37 mg/kg b.w. per day for nitrite (NTP, 2001).

Mutagenicity of nitrite was shown in vitro Salmonella typhimurium strain TA100 both with and without metabolic activation but not in strain TA98 (NTP, 2001). Chromosome aberrations in mammalian cells were investigated in in vitro culture of peripheral blood lymphocytes and at high concentrations of sodium nitrite (14.4 mM), a slight increase in micronucleated cells and chromatid gaps was noted (Balimandawa et al., 1993). No micronuclei induction in vivo in mice bone marrow or peripheral blood in a 14-week study was observed. Overall, the JECFA concluded that there is no evidence for the reclassification nitrite as a genotoxic compound (NTP, 2001; FAO/WHO, 2003a,b; EFSA, 2008a).

Three main endpoints have been identified regarding nitrite toxicity namely, methaemoglobinemia, hypertrophy of the adrenal zona glomerulosa (rat), and potential carcinogenesis (mouse):

(1) The acute formation of methaemoglobin (metHb) results from the reaction of the nitrite metabolite nitric oxide with oxyhaemoglobin, at the same time forming nitrate. Factors that are critical to metHb formation include increased nitrite levels, intestinal infection together with inflammation of the stomach lining and levels of nicotinamide adenine dinucleotide (NADH)-cytochrome b5 methaemoglobin reductase (which converts metHb back to haemoglobin). MetHb background levels are in the range of 1-3 %, above 10 % there is a reduction of oxygen transport, above 20 % cyanosis and hypoxia can occur, and an increase to 50 % can prove fatal (Mensinga et al., 2003). Infants under 3 months of age are more susceptible to metHb than adults due to a 40-50 % reduction of the activity of NADH-
cytochrome b5 methaemoglobin reductase together with their increased likelihood of getting intestinal infections (Savino et al., 2006).

(2) Hypertrophy of the adrenal zona glomerulosa has been investigated in a 13-week Wistar rat study, for which the no observed adverse effect level (NOAEL) was 5.4 mg/kg b.w. per day for the nitrite (Til et al., 1997; Boink et al., 1998; Mensinga et al., 2003). This finding is not considered pertinent to the current risk assessment of livestock feed.

(3) Two year carcinogenicity studies for sodium nitrite were conducted under the National Toxicology Programme (NTP, 2001) in B6C3F1 mice and F344/N rats. Mice (50 male and 50 female B6C3F1 mice) were exposed through drinking water to daily doses equivalent to 0, 60, 120, or 220 mg/kg b.w. per day (corresponding to 40, 80, 147 mg/kg b.w. per day for nitrite) and 0, 45, 90, or 165 mg/kg b.w. per day respectively (corresponding to 0, 30, 60, 111 mg/kg b.w. per day for nitrite). There were no differences in survival between exposed groups versus controls although lower body weights in females at the highest dose were observed. Incidences of squamous cell papilloma or carcinoma (combined) in the forestomach of female mice occurred with a positive dose-related trend but it was not statistically significant) with respective frequencies of 1/50, 0/50, 1/50 and 5/50 at 0, 45, 90, or 165 mg/kg b.w. per day respectively. In males, the incidence of hyperplasia of the glandular stomach epithelium was significantly greater at the highest dose and the authors concluded that there was equivocal evidence for carcinogenic activity in females based on the trend in the combined incidence of squamous cell papilloma and carcinoma of the forestomach (NTP, 2001).

In an early chronic toxicity study (Gruener and Shuval,1973), rats were given nitrite in the drinking water at doses equivalent to 0, 10, 100, 200 and 300 mg/kg b.w. per day (corresponding to 0, 6.7, 67, 124, 201 mg/kg b.w. per day for nitrite). No significant differences were found between control and treated groups for growth, mortality and total haemoglobin levels. At the highest three doses, methaemoglobin increased to 5, 12 and 22 % and lung toxicity was observed with dilatation of the bronchi with infiltration of lymphocytes and emphysema and at the highest dose, focal degeneration and fibrosis of the heart muscle as well as dilatation of coronary arteries were also observed. Based on heart and lung toxicity the NOAEL for sodium nitrite was 10 mg/kg b.w. per day and hence the NOAEL for the nitrite ion was 6.7 mg/kg b.w. per day (FAO/WHO, 2003a, b; EFSA, 2008a).

For nitrate, no adverse effects were observed in two dogs after dosing sodium nitrate in the diet at a level of 2 % for 105 and 125 days (Lehman, 1958), calculated to be equivalent to 500 mg/kg b.w. per day corresponding to 370 mg/kg b.w. per day nitrate (Walker, 1990).

The recent risk assessment performed by the CONTAM Panel used the NOEL value of 6.7 mg/kg b.w. per day of nitrite based on heart and lung toxicity from a 2 year rat study (Maekawa et al., 1982) and endorsed the ADI of 0-0.07 mg/kg b.w. per day for nitrite (FAO/WHO, 2003a,b; EFSA, 2008a). For nitrate, a NOEL of 500 mg/kg b.w. per day of sodium nitrate corresponding to 370 mg/kg b.w. per day of nitrate derived from long term studies in rats and a subchronic toxicity study in dogs (Lehman, 1958 cited in FAO/WHO 1962; Lijinski, 1973; EFSA, 2008a) was used to develop the ADI. A transposed NOAEL for nitrate, reflecting the conversion of nitrate to nitrite in the saliva and based on the NOAEL for nitrite were also considered by the JECFA (FAO/WHO, 2003a, b) and the recent CONTAM assessment for nitrate in vegetables. These values were derived by applying an uncertainty factor of 50 to the “transposed” NOAEL for normal converters of 160 mg/kg b.w. per day. This resulted in an ADI of 3.2 mg/kg b.w. per day for transposed nitrate which lies within the
same range as the traditionally established ADI for nitrate (3.7 mg/kg per day) (FAO/WHO, 2003a; EFSA, 2008a), thus supporting the latter figure.

2. Methods of analysis

According to Article 11 of Regulation No 882/2004 analysis methods used in the context of official controls shall comply with relevant Community rules or, (a) if no such rules exist, with internationally recognised rules or protocols, for example those that the European Committee for Standardisation (CEN) has accepted or those agreed in national legislation; or, (b) in the absence of the above, with other methods fit for the intended purpose or developed in accordance with scientific protocols.

Commission Regulation (EC) No 152/2009 of 27 January 2009 lays down the topical methods of sampling and analysis for the official control of feed. Contrary to a number of other undesirable substances, no official analytical method is prescribed by this Regulation for the determination of nitrite in animal feed.

The European Committee of Standardization (CEN) has elaborated a number of standards for the determination of nitrate and/or nitrite content of vegetables, vegetable products, including vegetable containing food for babies and infants as well as in meat and meat products (EN (12014), 1997a, b, c; 1998, 1999, 2005a, b). In contrast, no specific methods for the determination of nitrite in feed were established by CEN.

The official method of analysis of the Association of Official Analytical Chemists (AOAC) for the determination of nitrate and nitrite in animal feed is a colorimetric method. Nitrate and nitrite are extracted with cadmium chloride and barium chloride solution. Bulks of soluble proteins are precipitated in alkaline solution and the clarified solution is passed through a metallic cadmium column, reducing nitrate to nitrite. Nitrite is measured colorimetrically after a diazo-coupling reaction (Griess reaction) at 540 nm (AOAC, 1995). Many alternative methods have been developed and proposed, mainly in view of the toxicity and carcinogenicity of the cadmium column, used in the preparation of the samples. However, not all of them have been adequately validated but only compared with the above AOAC method with regard to some particular parameters such as the recovery and limits of detection and/or quantification.

Several methods have been reported for the simultaneous quantitative determination of nitrate and nitrite, including chromatography (Butt et al., 2001; Siu and Henshall, 1998; McMullen et al., 2005; Stalikas et al., 2003; Merino et al., 2000; Di Matteo et al., 1997), amperometry, capillary electrophoresis (Öztekin et al., 2002) and spectrophotometry (Ensafi et al., 2004; Casanova et al., 2006; Andrade et al., 2003; Kazemzadeh and Ensafi, 2001).

Methods, selectively intended for the quantitative determination of nitrite alone include kinetic methods (Koupparis et al., 1982; Liang et al., 1994), spectrophotometry with or without flow injection analysis (Ensafi and Dehaghei, 1999; Ghasemi et al., 2004; Fang et al., 2002; Chen et al., 1999; Ensafi and Keyvanfard, 1994), chemiluminescence (He et al., 2007; Gao et al., 2005), fluorescence (Li et al., 2003; Jie et al., 1999; Jie et al., 1994; Jie et al. 1993; Gao 2002), optical sensor technology (Kazemzadeh, 2005; Kazemzadeh and Daghmhi, 2005) and even dipstick technology (Fang et al., 2005).

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8 OJ L165, 30.4.2004, p. 1
9 OJ L 54, 26.2.2009, p. 1
3. Current legislation

The Council Directive 2002/32/EC prescribes that all products intended for animal feed must be of sound, genuine, and of merchantable quality and should neither pose risk to animal health and productivity nor to human health or the environment. Annex 1 to Council Directive 2002/32/EC\(^\text{10}\) lists at present a number of compounds that are considered undesirable in animal feeds and prescribes maximum limits in different feed commodities. The current EU maximum levels (ML) for certain feed materials containing nitrite are given in Table 1.

Table 1. EU legislation on sodium nitrite in feed materials as listed currently in Annex 1 of Council Directive 2002/32/EC.

<table>
<thead>
<tr>
<th>Undesirable substances</th>
<th>Product intended for animal feed</th>
<th>Maximum content in mg/kg relative to a feedingstuff* with a moisture content of 12 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite</td>
<td>Fish meal</td>
<td>60 (expressed as sodium nitrite)</td>
</tr>
<tr>
<td></td>
<td>Complete feedingstuffs* excluding:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- feedingstuffs intended for pets except birds and aquarium fish</td>
<td>15 (expressed as sodium nitrite)</td>
</tr>
</tbody>
</table>

*Complete feedingstuff: a mixture that is sufficient to fulfil the nutritional requirements for the animal species or category under consideration.

The sodium nitrite levels described in Table 1 correspond to a maximum content of the nitrite ion of 40 and 10 mg/kg in fish meal and complete feedingstuffs, respectively. Levels of nitrite in water are laid down in Council Directive 98/83/EC with a maximum limit value of 0.5 mg/l. With regard to 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 fertilizer and/or manures limiting concentrations of inorganic nitrogenous compounds in ground and surface waters. Council Directive 91/676/EEC has been implemented to protect waters from nitrate pollution in EU countries from agricultural sources. Maximum levels for nitrate in drinking water (50 mg/L) are also laid down in Council Directive 98/83/EC.

Nevertheless, it should be recognised that nitrite and nitrate are used as food additives particularly for their anti-bacterial properties against the potentially lethal pathogen *Clostridium botulinum*, and good endogenous efficacy against bacterial gastroenteritis (McKnight *et al.*, 1997; 1999; Duncan *et al.*, 1995; Dykhuizen *et al.*, 1996; Vallance, 1997).

4. Occurrence in feed

Animal rations may contain nitrite that is naturally present in vegetable feeds or less commonly which has been added as a preservative e.g. in the production of silage (it is used for silage for the same reason as in certain foods, aiming at the suppression of *C. botulinum*). Additionally, water can be an important source of nitrite via the reduction of nitrate.

\(^{10}\) OJ L 140, 30.5.2002, p. 10-22
4.1. Occurrence in feeding materials

4.1.1 Occurrence in plant feeds

The natural levels of nitrite in fresh plant material, with certain exceptions, are generally very low and few data have been published (Trif et al., 1986; EFSA, 2008a) and, as noted by SCAN (EC, 2003), typical levels in feedingstuffs have not been reported to cause toxicity in farm animals11. Nitrite is not normally present in soils to any significant extent, and as a result it is not normally available for uptake by plants. Furthermore, if nitrite would be present in significant quantities, it would be toxic to the plant (Archer, 1985). As in the case of vegetables grown for human consumption, several plant species are known to accumulate nitrate up to very high levels, and adverse health effects have been associated with more than 80 forage species that can lead to nitrite poisoning (Clarke and Clarke, 1975). Practically, the majority do not represent major feeds for livestock. Nitrite poisoning occurs predominantly in ruminants although pigs are potentially more susceptible to nitrite poisoning than cattle or sheep (Stormorken, 1953). The reason for the difference is that pigs are not normally fed forage-based diets which have the potential for higher nitrite content than the predominantly cereal-based feeds employed for pigs. Overall, the incidence of nitrite poisoning in livestock is generally low due to farmer awareness and their management of the known risk factors both for the animals and the feed supply/constituents with good agricultural practice. Table 2 reports the content of nitrate (the major source of nitrite via interconversion) of feeds which are generally considered to be safe as guidance values (Crowley, 1985).

Table 2. Typical nitrate content of common feeds dry matter basis) considered to be safe.

<table>
<thead>
<tr>
<th>Feeding stuff</th>
<th>NO₃ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize grain</td>
<td>22</td>
</tr>
<tr>
<td>Oat grain</td>
<td>44</td>
</tr>
<tr>
<td>Soybean oil meal</td>
<td>4</td>
</tr>
<tr>
<td>Fresh alfalfa or alfalfa hay</td>
<td>1760</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>880</td>
</tr>
</tbody>
</table>

The accumulation of nitrate in plants is influenced by a range of factors (Cooper and Johnson, 1984; Osweiler et al., 1985; Gupta, 2007):

- **Stage of maturity**: under normal growing conditions, nitrate concentrations tend to decrease as plants mature, and therefore young plants have higher nitrate concentrations than mature plants. However, mature plants can still have high nitrate concentrations if the species, environmental and soil conditions are favourable for nitrate accumulation.

- **Fertiliser application**: there is a direct relationship between the level of nitrogen fertiliser application and the nitrate content of the plants (Wehrmann, and Scharpf, 1989). Soils high in nitrogen readily supply nitrate to plants, and as a consequence soils which have been heavily fertilized/deep ploughed tend to produce crops with higher nitrate levels.

- **Growing conditions**: elevated levels tend to be found in crops growing in conditions that tend to limit the growth of the plant while still allowing for the uptake of nitrate. Drought, frost damage, cold temperatures, low light and treatment of crops with growth retarding

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11 Occasional adverse effects result from stored hay or silage if they are allowed to heat up or are contaminated by bacteria or fungi (Spoelstra, 1985, 1987).
herbicides and shading have all been shown to result in higher than normal nitrate levels in some forages.

- **Plant species:** more than 80 plant species, which include a number of common agricultural crops that have been associated with nitrite poisoning, can accumulate nitrate to high levels under extreme conditions. These include maize (*Zea mays* L. ssp. *mays*), rape (*Brassica napus*), soybean (*Glycine max*), linseed (*Linum usitatissimum*), sorghum (*Sorghum spp*), millet (widely cultivated species from the genera *Pennisetum*, *Setaria* and *Panicum*), wheat (*Triticum spp*), oats (*Avena sativa*) and barley (*Hordeum vulgare*) and most weeds such as lamb’s quarter (*Chenopodium album*), thistle (*Arctium, Carduus, Centaurea spp*), Jimson weed (*Datura stramonium*), fireweed (*Epilobium angustifolium*), smartweed (*Polygonum hydropiperoides*), dock (*Rumex spp*), and Johnson grass (*Sorghum vulgare*) (The Merck veterinary manual, 2006).

Because of this range of factors and particularly the impact of fertiliser application rates, levels of nitrate in forage crops can vary widely, but normally remain within safe ranges. To illustrate the impact for common feeds and forages of different fertiliser application rates and harvesting regimens, resultant nitrate levels are shown in table 3 (adapted from Cash *et al*., 2007).

### Table 3. Nitrate concentrations in common feeds and forages after different fertiliser or harvesting regimens.

<table>
<thead>
<tr>
<th>Fertiliser application rates or harvesting regimens</th>
<th>NO₃ (mg/kg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oat hay at soft dough, 67 kg/ha</td>
<td>2149</td>
</tr>
<tr>
<td>Oat hay at soft dough, 134 kg/ha</td>
<td>5613</td>
</tr>
<tr>
<td>Barley hay at soft dough, 67 kg/ha</td>
<td>868</td>
</tr>
<tr>
<td>Barley hay at soft dough, 134 kg/ha</td>
<td>2627</td>
</tr>
<tr>
<td>First crop alfalfa, vegetative</td>
<td>1800</td>
</tr>
<tr>
<td>Second crop alfalfa, vegetative</td>
<td>3200</td>
</tr>
<tr>
<td>Fresh chopped maize (before ensiling)</td>
<td>4400</td>
</tr>
</tbody>
</table>

* expressed on a dry matter (DM) basis

Levels of nitrate tend to be highest in the lower stems and leaves of plants, while very little is found in the seeds (table 2 and 3; Walker *et al*., 1990; MAFF, 1998). As a result, forage crops - rather than cereal grains or oilseed meals - represent the greatest risk of diets exceeding the maximum permitted levels of nitrite (10 mg/kg, expressed as the nitrite ion) as specified in Council Directive 2002/32/EC.

Environmentally, the most significant source of nitrate and the cause for much of the higher levels noted in forages and groundwater is from the nitrogen in animal manure and fertiliser. When animal manures and fertilisers are repeatedly applied to limited areas of land, nitrates tend to build up in the soil, leach to groundwater, and are taken up by the roots of plants and deposited in plant tissues. Under many conditions, nitrates are degraded by plant enzymes to forms of nitrogen that pose little hazard to either the animal or to the environment in general. Nitrates tend to be highest in the plant parts nearest the ground as well as in stalks, stems and leaves (Robinson, 1999; EFSA, 2008a).
4.1.2. Occurrence from use as a preservative

Nitrite is widely used as an approved food additive (Cammack et al., 1999; EFSA, 2008a) in human and pet food. It has also been used as an additive for the preservation of animal feed, in particular fish meal and forages.

4.1.2.1. Fish meal

The use of sodium nitrite as a preservative was introduced in European fisheries in the 1940’s because it was more effective than salt, which had been used until then. Initially the only recognised consequence of high levels of nitrite in feedingstuffs was a reduction of the content of vitamin A in the liver of consuming animals; no other adverse effects were reported at the time.

Subsequently, in the early 1960s, serious health effects were observed in livestock fed fish meal (Sakshaug et al., 1965; Koppang, 1974). The toxic substance was identified as N-nitrosodimethylamine (NDMA), which is also carcinogenic. The addition of nitrite to fishmeal was identified as one of the factors associated with NDMA production via interaction with amino acids. Juszkiewicz et al., (1980) analysed 465 samples of ingredients of fish meal, Antarctic krill meal and experimental silage containing 40 % dried animal wastes for nitrate and nitrite content in the period 1973-78. Over 62 % of the samples contained nitrates in concentrations ranging from 1 to 1020 mg/kg, and 6 % contained 1-15 mg/kg of nitrites. Of 171 selected samples, 40 % were found to contain also NDMA in the range of 0.003 to 0.417 mg/kg but no statistical correlation between concentrations of nitrates or nitrites and nitrosamines could be confirmed. Precursors, such as dimethylamine, have also been found in large amounts ranging from 110 to 1765 mg/kg, in all samples of krill meal (Juszkiewicz et al., 1980).

An alternative method of preserving fish was developed, which involved the use of a combination of sodium nitrite and formaldehyde, and studies showed that this reduced NDMA levels significantly. However, its use declined and in 1993/94 the use of sodium nitrite as a preservative was forbidden. Today, preservation of fish caught at sea is achieved by a combination of the use of refrigerated seawater and acetic acid. As a result of changes in the method of preserving fish, analysis of nitrite in fishmeal is no longer given a high priority, and therefore limited data are available on current levels of nitrite in fishmeal. However in the absence of exogenously applied nitrite for preservation, the levels in fresh fish and derived fish meals are likely to be low (Andrew Jackson, International Fishmeal and Fish Oil Organisation, personal communication).

4.1.2.2. Forages

Fresh forage crops such as maize, grasses, legumes, wheat and alfalfa can be preserved by ensiling, and are highly valued as animal feed. In order to preserve the crop successfully, it is important to achieve a good microbial fermentation. Frequently a chemical or microbial additive is applied to the crop at harvesting to improve the fermentation process, either to assist or augment the microorganisms that are naturally present, or restrict the activity of undesirable micro-organisms. This latter group include Enterobacteriaceae, which compete with beneficial bacteria for nutrients while reducing the quality of the protein in the forage. Sodium nitrite has been used as a forage additive to control these bacteria (Woolford et al., 1978), but as a result of the development of more effective forage additives sodium nitrite is not now widely used.
The formation of volatile and non-volatile nitrosamines may also naturally occur in feeds other than fish meals when sodium nitrite is added as preservative (Juszkiewicz, et al., 1980). The fermentation in silages of naturally nitrate rich forages (such as Lollium perenne), may lead to the formation of NMDA and nitrosodiethylamine up to 2 µg/kg DM, With the stabilisation and the ventilation of the silages, the concentration of the most representative nitrosoamines is normally nearer to 0.6 µg/kg DM (van Broekhoven and Davies, 1980).

4.1.3. Occurrence in water

Levels of nitrite in water are laid down in Council Directive 98/83/EC with a maximum limit of 0.5 mg/L (EC, 1998).

Water is also a potentially significant source of nitrite for livestock via reduction of nitrate. Nitrate is extremely soluble in water and can move easily through soil into the drinking water supply. As a result, nitrate movement into surface and ground waters is of concern both for health and environmental quality reasons (Galloway et al., 2003). Although naturally present in groundwater, elevated levels usually result from human activities such as overuse of chemical fertilizers or manures, improper disposal of human or animal excreta, and certain industrial processes. These fertilizers and wastes are sources of nitrogen, containing compounds which are converted to nitrate in the soil. According to a European Commission report (COM 120, 2007) on the implementation of Council Directive 91/676/EEC regarding the protection of waters from nitrate pollution in 15 countries of the EU from agricultural sources for the period 2000-2003, it is not unusual for nitrate levels, to exceed 100 mg/L in groundwater, despite the upper EU limit set by the Drinking Water Directive of 50 mg/L.

4.1.4. Nitrite and Nitrate in feed - Data from Member States

In order to understand today’s levels of exposure to nitrite by farmed livestock and fish within Europe, European countries were invited to provide information on levels of nitrite and nitrate in feeding-stuffs acquired as part of routine surveillance programmes. Data on levels of nitrite in 94 samples of feed were received from 3 European countries for the period 2002-2008. Slovenia was the only country also providing data on levels of sodium nitrate in 22 samples of feed for the period 2003-2007. Details of the origin of the samples are given in Table 4.

The analytical method used by the different Member States and the corresponding limits of detection (LODs) and limits of quantification (LOQs) are reported in Table 5.

Table 4. Origin and numbers of samples of animal feeding-stuffs analysed for nitrite and nitrate in the period 2002-2008 as reported by Member States.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Country</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite</td>
<td>Cyprus</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>4</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>12</td>
<td>6</td>
<td>1</td>
<td>8</td>
<td></td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slovenia</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>12</td>
<td>13</td>
<td>20</td>
<td>18</td>
<td>9</td>
<td>15</td>
<td>7</td>
<td>94</td>
</tr>
<tr>
<td>Nitrate</td>
<td>Slovenia</td>
<td>3</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td></td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Method of analysis for nitrite and nitrate and limits of detection (LOD) or limits of quantification (LOQ) per country.

<table>
<thead>
<tr>
<th>Country</th>
<th>Occurrence data reported for</th>
<th>Method of analysis</th>
<th>LOD (mg/kg)</th>
<th>LOQ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>Nitrite</td>
<td>Spectrophotometry (Analyse par spectrométrie d'absorption moléculaire; Issue de la norme pour le dosage des nitrites dans les eaux NF EN 26777).</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Slovenia</td>
<td>Nitrite / Nitrate</td>
<td>AOAC 968.07, 1995, Ch. 4, p. 14</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Tables 6 and 7 provide summary statistics (average, median and maximum values), for each country and for each of the main feed commodity groups, of the data provided for nitrite and nitrate content, respectively. In the same tables, information on the number of samples that exceeded the LOD or the LOQ and, in the case of nitrite, above the permitted MLs, was also reported. Only 23 samples out of 94 exceeded the LODs or LOQs, no samples above the MLs for nitrite were detected. The highest levels of nitrite and nitrate were detected in fodder in Slovenia, of 6.7 and 58.4 mg/kg on average, respectively.

Table 6. Total levels of nitrite in mg/kg reported by country for main feed commodities in comparison with maximum limits.

<table>
<thead>
<tr>
<th>Feed commodity group</th>
<th>Country</th>
<th>ML nitrite (mg/kg)</th>
<th>Mean (mg/kg)</th>
<th>Median (mg/kg)</th>
<th>Maximum (mg/kg)</th>
<th>No &gt; LODs or LOQs</th>
<th>&gt; MLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complementary feed*</td>
<td>Slovenia</td>
<td>-</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Complete feed</td>
<td>Slovenia</td>
<td>10</td>
<td>2.5</td>
<td>1.6</td>
<td>7.9</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Fish - Complete feed</td>
<td>Cyprus</td>
<td>-</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Fishmeal</td>
<td>Cyprus</td>
<td>40</td>
<td>1.6</td>
<td>0.3</td>
<td>11.3</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>40</td>
<td>1.4</td>
<td>1.0</td>
<td>11.2</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Slovenia</td>
<td>40</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Forage</td>
<td>Slovenia</td>
<td>-</td>
<td>6.7</td>
<td>3.5</td>
<td>26.2</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Other feed</td>
<td>Slovenia</td>
<td>-</td>
<td>2.6</td>
<td>1.7</td>
<td>6.5</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>94</strong></td>
<td><strong>23</strong></td>
<td><strong>0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MLs: Maximum limit for nitrite in feed (mg/kg); Mean; mean value for nitrite in feed (mg/kg); Median: median value for nitrite in feed (mg/kg); Maximum: maximum value for nitrite in feed (mg/kg); No: Number of samples analysed for nitrite in feed (moisture content of 12%) in the period 2002-2008; > LOD or LOQs: Number of samples that exceeded the Limit of Detection (LOD) or Limit of Quantification (LOQ); > MLs: Number of samples that exceeded maximum Limits (MLs).

*Complementary feed: a mixture with a high content of certain substances, which, because of their composition, is only sufficient for a daily ration if used in combination with other feedingstuffs.
Table 7. **Total levels of nitrate in mg/kg reported by Slovenia for main feed commodities.**

<table>
<thead>
<tr>
<th>Feed commodity group</th>
<th>Mean</th>
<th>Median</th>
<th>Maximum</th>
<th>No. of samples</th>
<th>&gt; LODs or LOQs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complementary feed</td>
<td>31.3</td>
<td>31.3*</td>
<td>47.3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Complete feed</td>
<td>8.6</td>
<td>7.9*</td>
<td>19.6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Forage</td>
<td>58.4</td>
<td>1.8</td>
<td>394.1</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Other feed</td>
<td>13.0</td>
<td>1.8</td>
<td>40.6</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>22</td>
<td>12</td>
<td>22</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Mean: mean value for nitrate in feed (mg/kg); Median: median value for nitrate in feed (mg/kg); Maximum: maximum value for nitrate in feed (mg/kg); *Median values based on only 2 samples; No: Number of samples analysed for nitrate in feed (moisture content of 12%) in the period 2003-2007; >LOD or LOQs: Number of samples that exceeded the Limit of Detection (LOD) or Limit of Quantification (LOQ).

5. **Estimating nitrite intake in feed by farm livestock**

Exposure to nitrite has been estimated in livestock based on the maximum authorised levels of nitrite in feed (10 mg/kg). Water, as a source of nitrite, has also been estimated using the EU maximum limit of 0.5 mg/L to illustrate its possible contribution in comparison with feed. Estimates of feed intake are based on typical feeding regimens within Europe that have formed the basis of previous EFSA exposure estimates in livestock. Table 8 illustrates the likely exposure of monogastric livestock to nitrite given complete feed and according to the above assumptions.

Table 8. **Estimated exposure of monogastric livestock to nitrite from feed and water given a diet containing the maximum permitted sodium nitrite concentration (15 mg/kg) expressed as nitrite ion (10 mg/kg) and water at the EU maximum limit value.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Live weight</th>
<th>Consumption</th>
<th>Nitrite intake from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kg</td>
<td>total complete feed</td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td>kg/day</td>
<td>l/day</td>
<td>mg/kg b.w. per day</td>
</tr>
<tr>
<td>Pigs</td>
<td>100</td>
<td>3.7</td>
<td>0.37</td>
</tr>
<tr>
<td>Sows</td>
<td>250</td>
<td>6.5</td>
<td>0.26</td>
</tr>
<tr>
<td>Poultry (broilers)</td>
<td>2.1</td>
<td>0.15</td>
<td>0.71</td>
</tr>
<tr>
<td>Poultry (laying hens)</td>
<td>1.9</td>
<td>0.115</td>
<td>0.61</td>
</tr>
<tr>
<td>Fish</td>
<td>4.5</td>
<td>0.09</td>
<td>0.20</td>
</tr>
</tbody>
</table>

^1 Nitrite in total complete feed assumed equal to 10 mg/kg; ^2 Water contribution using the EU maximum limit value of 0.5 mg/L is given for comparative purposes.

Table 9 illustrates the predicted exposure of ruminants to nitrite given compound feed containing the maximum permitted nitrite concentration (10 mg/kg), forage at the maximum value reported by Slovenia (26.2 mg/kg) (Table 6), and water at the EU maximum value for nitrite (0.5 mg/L).
Table 9. Estimated exposure of ruminants to nitrite from feed and water where the diet contains the maximum permitted sodium nitrite concentration (15 mg/kg) in compound feed and the maximum reported concentration in forage, expressed as nitrite (Table 6). These calculations assume typical livestock feed and represent a worst-case scenario.

<table>
<thead>
<tr>
<th>Species</th>
<th>Live weight</th>
<th>Consumption</th>
<th>Nitrite intake from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kg</td>
<td>Forages</td>
<td>Compound feed</td>
</tr>
<tr>
<td>Dairy cow</td>
<td>625</td>
<td>14.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Suckler cow</td>
<td>550</td>
<td>11.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Cattle</td>
<td>300</td>
<td>7.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lactating ewe</td>
<td>70</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Growing lamb</td>
<td>20</td>
<td>0.45</td>
<td>0.15</td>
</tr>
<tr>
<td>Dairy goats</td>
<td>65</td>
<td>1.50</td>
<td>0.7</td>
</tr>
</tbody>
</table>

ML = Maximum limit; DM = Dry Matter; \(^1\) Nitrite intake from forages using the maximum value of 26.2 mg/kg DM reported by Slovenia (Table 6); \(^2\) Nitrite intake from compound feed, using 11.36 mg nitrite/kg DM, (10.0 mg/kg for a feed with a moisture content of 12 %); \(^3\) Nitrite in water assumed equal to 0.5 mg/l.

Exposure to nitrite in grazing animals may also arise from soil ingestion and such exposure has been estimated to vary between 1 – 18 % of the feed dry matter intake (Thornton and Abraham, 1983).

Nitrate concentrations in common feeds and forages after different fertiliser and harvesting regimens can be high (Table 3). Overall, inter-conversion of nitrate constitutes the major source of nitrite intake in farm animals particularly in ruminants. However, the limited data reported from Slovenia (table 7) showed that the maximum nitrate levels were more than 10-fold lower and ranged from 20 mg/kg in complete feed to 394 mg/kg in forages.

Considering the evidence presented in Section 6 below, the most sensitive species of livestock are pigs and cattle (as ruminants). The overall nitrite exposure from feed was found to be 0.37 mg/kg nitrite b.w. per day in pigs (Table 8) and 0.65 mg/kg nitrite b.w. per day in cattle (Table 9). Such exposure would rise to 0.42 and 0.70 mg/kg nitrite b.w. per day in pigs and cattle respectively taking into account water intake at the maximum EU limit value of 0.5 mg/L.

6. **Adverse effects in livestock, fish and pets**

Feed and water constitute the main source of exposure both to nitrite and nitrate in animals, which at excessive levels may cause adverse effects in livestock, fish and pets (Stoltenow and Lardy, 1998). The main acute toxicity endpoint of excess nitrite in a wide range of farm animals (cattle, sheep, swine, dogs, chickens and turkeys) is methaemoglobinemia, especially in the young. Not only is the acute toxicity of nitrate approximately ten-fold less than that of nitrite, but there is also a general consensus that this 10-fold ratio extends to the potential for methaemoglobin formation (NIOSH, 1987). Whereas nitrite is active directly, nitrate has to be inter-converted to nitrite and this kinetic aspect helps to explain why higher levels of dietary nitrate intake cannot simply be predicted to add to the nitrite burden per se.
In monogastric animals, with the exception of rats and mice, an important source of nitrite is dietary nitrate that is systemically absorbed, secreted into the saliva and then reduced to nitrite via nitrate reductase activity due to bacteria in the oral cavity and tongue (Bruning-Fann and Kaneene, 1993; Djekoun-Bensoltane et al., 2007). Such conversion is pH (alkali) dependent and therefore does not normally occur in the stomach of most monogastric animals due to relative acidity (pH < 3.5) (Wright and Davison, 1964; Mirvish, 1975). In contrast, the rumen of ruminants and the enlarged caecum and colon of horses are especially suited for nitrate reduction due to the dense microbial population and a relatively high pH (> 5) (Wright and Davison, 1964; Sen et al., 1969; Mirvish et al., 1975). Since herbivorous species, and in particular ruminants, feed on diets containing high levels of nitrate they are potentially one of the most sensitive species to nitrite poisoning due to their innate ruminant ability for microbial conversion of nitrate to nitrite. However, under normal circumstances problems do not occur as the resultant nitrite is rapidly removed via bacterial fermentation for anabolism to produce ammonia, amino acids and proteins.

\[
\text{Nitrate} \rightarrow \text{Nitrite} \rightarrow \text{Ammonia} \rightarrow \text{Amino acids} \rightarrow \text{Proteins}
\]

An important aspect for the chronic ingestion of nitrate and nitrite in ruminants is the progressive and relatively rapid adaptation of the gut and the flora of the rumen, which aids tolerance by increasing the detoxification of nitrite to ammonia under different loadings. For this dynamic reason, a reproducible conversion factor is not available. In certain circumstances nitrite accumulation can occur when nitrate is converted to nitrite faster than nitrite is converted to ammonia. Consequently, when exceptionally high amounts of nitrate (and potentially nitrite) are consumed, an overload of nitrite may occur in the rumen. Nitrite is then absorbed into the bloodstream, which, via nitric oxide metabolites, converts haemoglobin to methaemoglobin (Bruning-Fann and Kaneene, 1993).

The susceptibility to nitrate-nitrite intoxication in animals is also modulated by the activity of the nicotinamide adenine dinucleotide phosphate (NADPH) dependent reductase, an erythrocyte cytosolic and membrane enzyme that converts methaemoglobin back to haemoglobin. Its relative activity, shows large inter-species variability and has been estimated as a percentage of the activity of the human form for pigs (27 %), horses (63 %), cattle, cats and goat (90 %), dog (114 %), sheep (150 %), and rabbit (452 %) (Kuehnert, 1991): this helps to explain the relative sensitivity of pigs. Intra-species differences, particularly in different breeds of dogs, cats and horses, have been shown together with congenital defects in enzyme. Age-related differences in the activity of this enzyme have also been reported particularly in neonatal and old animals (Harvey, 2006).

Another factor affecting the susceptibility to nitrite exposure in foetuses and neonatal animals is the expression of foetal haemoglobin, which has a higher affinity for oxygen and hence forms methaemoglobin more readily compared to the adult form (WHO, 2007).

Thus, nitrite/nitrate intoxication in animals is generally related to different exposure scenarios, together with age and digestive tract differences between monogastrics, ruminants, caecal/colonics, rodents, and differing physiological sensitivity resulting from differences in methaemoglobin reductase activity. It is important to note that nitrate levels are relatively high in stalk and leaves compared to seeds, and animals mainly fed on forages are thus exposed to nitrate intakes to a higher extent than animals fed on seeds. As a consequence,
poultry and to a lesser extent pigs, gain negligible nitrite/nitrate intake from feed, and the larger dietary contribution is from water (Bruning-Fann and Kaneene, 1993).

Endogenous production of nitrite/nitrate has been reported in cattle as result of bacterial endotoxin exposure since the latter induces nitric oxide synthase. This effect has been shown to be enhanced when arginine supplementation is used in feeds and as then blood nitrate levels can be raised from <5 up to 9 NO3 µmol/L (Hüsler and Blum, 2001).

The observed adverse effects of nitrite in livestock range from acute to chronic, with various degrees of severity. Clinical signs of acute toxicity predominate and, with symptoms such as accelerated pulse, dyspnoea, muscle tremors, weakness, vomiting, unstable gait, cyanosis and death, are generally due to methaemoglobinemia. Signs of sub-chronic and chronic toxicity include reduction in feed intake, reduction of milk production in dairy animals, rough hair, weight loss or no weight gain, Vitamin A (in rat, pigs, poultry, sheep) (WHO, 2007) and vitamin E reductions, reduced fertility and abortion. Abortion has been correlated with foetal hypoxia due to methaemoglobinemia, low fertility has been associated with nitrite-induced deficiency in vitamin A, and reduced steroidogenesis via mitochondrial nitric oxide production, alteration of cytochrome P450 enzyme activity gene expression and thus transcription or translation of the steroid acute response protein (SARP) (Guillette and Edwards, 2005). Anti-oxidants (ascorbic acid, methionine and vitamins A and E) reduce nitrite toxicity, and are thought to trap possible nitrogen free radicals produced as a consequence of nitrite and/or nitrate metabolism; on the other hand, pro-oxidant substances may enhance nitrite induced toxicity. Goitrogenicity of nitrite has been reported only in poultry whereas nitrate has been shown to have this effect on different species i.e. swine, sheep, cattle, resulting from inhibition of iodine uptake from the thyroid gland due to nitric oxide and reduced activation of the cyclic guanosine monophosphate (cGMP) pathway involved in thyroid hormone bio-synthesis (Bazzara et al., 2007).

The severity of the intoxication from nitrite exposure may also depend on other factors such as dietary status, sudden changes in diet composition (i.e. from grains to leafy forages) or the presence of metal ions and sulphur compounds in the diet that may enhance/inhibit the reduction of nitrate to nitrite.

Antibiotics may also influence the salivary and in general gastro-intestinal formation of nitrite from nitrates due to their impact on the bacterial flora: broad spectrum antibiotics formerly used in animal production have been proven to limit nitrite formation, with possible consequences on mucosal defences against fungal and bacterial pathogens (Dougall et al., 1995). On the other hand, the administration of formerly EU licensed ionophoric polyethers Monensin and Lasalocid (Rumensin® and Bovatec®) as feed additives (currently in use in the USA) respectively, increased the production of nitrite in the rumen with the potential to lead to intoxication (Malone, 1978; Rogers and Jope-Cawdery, 1980; Slenning et al., 1991; Goelz, 2002).

Although the legislation expresses maximum nitrite content in mg/kg relative to a feedingstuff with a moisture content of 12 %, to compare published studies and to calculate exposure on a body weight basis, all the following data have been standardised to nitrite on a 100 % dry matter basis.

Viewed overall for livestock, differences in gastro-intestinal physiology between monogastric and ruminants, together with a wide variety of husbandry practices, dietary regimens and possible aggregate exposures from soil, water, feed and forages, do not allow the derivation of
clear dose-response relationships for the toxicity of nitrite and nitrate in livestock, as is the case for well controlled laboratory animal studies.

Moreover, literature reports of livestock intoxication due to nitrite are scarce, and most scientific publications have either been devoted to nitrate or to combined nitrate and nitrite exposures. Because of the inter-conversion of nitrate to nitrite studies involving nitrate have also been included in the following sections to illustrate the potential for adverse effects both qualitatively and quantitatively and to help fill data gaps where no data on nitrite exists. Some studies involved feedingstuffs containing endogenous nitrite or nitrate whereas other studies involved the dosing of the chemicals per se. The findings were then used to approximate the lowest observed adverse effect levels (LOAELs) for the direct nitrite content of the diet. Where no nitrite exposure data existed nitrite was estimated on the basis of nitrate exposure assuming a 10-fold ratio in acute toxicity between nitrite and nitrate. NOAELs were also estimated for the different livestock species using an uncertainty factor of 3 to convert LOAELs to NOAELs. Where lethal dose (LD$_{50}$) data are the only figures available, the LOAEL was estimated to be 10% of the LD$_{50}$ value.

6.1. Ruminants

General aspects

Nitrite poisoning in ruminants is uncommon. Moreover, the threshold levels for nitrite/nitrate in feed able to cause acute and/or chronic adverse effects in animals are difficult to determine because of the difficulty in tracking all sources of nitrite exposure, including inter-conversion from nitrate. Breed sensitivity, age, health and physiological status, the timing and length of exposure and the progressive adaptation of the gastro-intestinal flora to high levels of nitrite/nitrate in feed are also important factors to consider when assessing toxicity of nitrite (Brunning-Fann and Kaneene, 1993).

Nitrate contained in milk replacers fed to calves (monogastric at this age) can be converted to nitrite in the stomach if the pH of the gastric fluid is sufficiently high (above pH 5). Calves exposed to water that is highly contaminated with bacteria are particularly sensitive to nitrite/nitrate toxicity. Drinking water containing both high nitrate levels and significant coliform contamination (> 10 colony-forming units (CFU)/100 mL), able to reduce nitrate to nitrite has a greater potential to affect health adversely and to lower productivity than do either nitrate or bacteria alone. A high-producing dairy cow may drink over 120 L/day of water when farmed at + 27°C (Grant, 2000; Waldner and Looper, 2001). Silages and hay may contain over 200 mg/kg nitrite on a dry weight basis as “background levels” and close monitoring by farmers is normal to prevent outbreaks of intoxication (van Broekhoven and Davies, 1982; Strickland et al., 2008). On the basis that silages and hay may account for 50% or more of the dry feed intake in ruminants, a theoretical complete feed for such animals could considerably exceed the regulatory limit of 9.15 mg/kg nitrite on a 12% moisture basis corresponding to 10.1 mg/kg on a dry matter basis, even if the nitrite contribution from complementary feeds was negligible. However as shown in Table 2, the levels that are generally considered to be safe for mixed feeding do in fact considerably exceed these “background level” figures showing the conservative nature of this estimate compared with the farm experience.

Adult cattle

No specific studies are available that report quantitatively nitrite exposure and toxicity in adult cattle although a range of studies refer to nitrate intoxication and methaemoglobinemia.
The LD₅₀ for nitrate in cattle has been estimated by Bradley et al. (1940) as 330 mg/kg b.w. when administered via drenching; compared with 990 mg/kg b.w. for exposure from feed. Nitrate toxicity was reported in cattle fed on maize stalks, oat straw or weedy pasture and according to occurrence data from 803 forages analysed during outbreaks of death in Oklahoma, the mean nitrate levels in the feed ranged from 6,000 to 14,000 mg/kg dry weight (Edwards and McCoy 1980). Rogers (1999) suggested that feeds with 5600-9400 mg/kg nitrate dry matter should be restricted to <25% of the total ration for cattle and that water levels higher than 220 mg nitrate/L should be avoided. Water levels >656 mg nitrate/L should be regarded as lethal. For growing cattle, with a water intake of 30 L per day and a feed intake of 2 kg dry weight per day (25% intake as recommended above), total nitrate levels of 6,600 mg in water and 11,200 mg in feed can be estimated. This would approximate to 37.3 mg/kg b.w. per day for a 300 kg growing cattle from feed intake only.

**Dairy cows**

Adverse effects data on nitrite poisoning in dairy cows are scarce and most of the literature available is on nitrate or combined exposure from nitrite/nitrate.

**Nitrate and Nitrite data:** stillborn or blind calves showing hydroperitoneum, lung oedema, ascites and haemorrhages in the gastro-intestinal system of four calves at autopsy were described as a consequence of chronic cumulative exposure to nitrite and nitrate in feeds and water of pregnant dairy cows. Levels of exposure in these cows ranged from 685 to 2,136 mg/kg DM and from 19.7 to 186 mg/L, for nitrate, and from 4.9 to 65.8 mg/kg DM and from 1.1 to 5.4 mg/L for nitrite, respectively. Possible confounding effects, for instance the possibility of birth defects were considered and then excluded (Ozmen et al., 2005). Hence, for a 625 kg lactating dairy cow, with a daily intake of 24 kg DM and 120 L water, the lowest average level of nitrate exposure would result in an overall intake of 30 and 72 mg/kg b.w. per day from both feed and water sources. However, the authors discussed that the observed toxicity was due to chronic exposure to high nitrate levels which were approximately 100-fold above the nitrite level on a daily and body weight basis (Ozmen, 2005). Hence, a LOAEL for nitrite can not be derived from this study.

**Nitrate data:** abortion in pregnant cows has been observed sub-acutely between 2-21 days after the onset of exposure to nitrate at levels of 3,000 mg/kg DM in feed corresponding to approximately 115 mg/kg b.w. per day. Some cows, which aborted, did not show any other signs of poisoning. In other cases reports of prolonged calving, stillbirth and birth of weak (soft) calves as consequence of acute, sub-acute or chronic nitrate poisoning in cows were also recorded and correlated with high levels of methaemoglobinemia recorded in still-born calves. Overall, the authors concluded that abortion was caused by a general state of hypoxia of the foetus, determined by the extensive methaemoglobin formation in the blood of the pregnant cow and foetus (Brunning-Fann and Kaneene, 1993). Haliburton and Edwards (1978) reported an abortion rate of 12.6% on average in cows exposed to a mean 800 mg/kg DM nitrate in contaminated forages corresponding to approximately 30 mg/kg b.w. for a 625 kg cow, assuming a 24 kg DM intake.

**Calves**

No studies on nitrite per se are available in calves.

**Nitrite and Nitrate data:** methaemoglobin formation and cyanosis were observed in calves (75 kg) after an oral administration in an aqueous solution of 2,600 mg and 18,300 mg of nitrite.

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12 Calculated using body weight of 625 kg a feed intake of 24 kg DM and 3 000 mg/kg DM nitrate in feed.
Nitrite and nitrate corresponding to 34 mg/kg b.w. and 244 mg/kg b.w. per day respectively (Baranova et al., 2000). In a previous study, no signs of toxicity were noted in animals exposed for six weeks to 1000-5000 mg per day nitrate intake via drinking water (Baranova et al., 1999).

**Nitrate data:** nitrate levels of 320 mg/kg b.w. nitrate (equivalent to 10,000 mg/kg DM in feeds) consumed in feed over 4 hours, have been shown to cause adverse effects whereas a dose of 990 mg/kg b.w. was tolerated when consumed over 24 hours. During an 8-week study, 75 kg calves (12 males/group) receiving 1 kg artificial milk containing 5 – 133 mg/kg b.w nitrate per day (18 (control), 400, 2,000, 5,000 or 10,000 mg/kg in artificial milk) did not show adverse effects (growth pattern, weight gain, food conversion, biochemical blood parameters, or morphology of the liver and kidneys) (Berende et al., 1977). These findings can be related to the lack of quantitative reduction of nitrate to nitrite in non-ruminant calves.

**Sheep**

As previously discussed for cows, the progressive adaptation of rumen flora to high nitrate content in forages and feeds influences the onset and gravity of the clinical symptoms seen in sheep. The majority of the following data relate to nitrite administration per se rather than as a component of feeding stuffs.

**Nitrite data:** Bartik and Piskac (1981) reported an oral lethal dose of nitrite in the range of 67-110 mg/kg b.w. Trif et al. (1993) showed that after a single oral administration of 83 mg/kg b.w. death occurred in all sheep while a single 50 mg/kg b.w. dose resulted in a rise of methaemoglobinemia up to 10% at 4 hours from administration. The same nitrite dose administered over 7 days did not induce clinical signs of intoxication but the methaemoglobin concentration increased to 5.9%. Over sixty days of treatment, a 10 mg/kg dose was still able to increase methaemoglobin concentration to 7%.

**Nitrate and nitrite data:** Sinclair and Jones (1967) showed that single oral doses of nitrite at 64 mg/kg b.w. (approximately equivalent to 2,100 mg/kg DM) and 224 mg/kg b.w. nitrate (roughly equivalent to 7,500 mg/kg DM) were lethal. However equivalent doses were tolerated when administered over 24 hours; a situation more akin to normal dietary intake, excluding the feed matrix and thus still reflecting a worst-case scenario.

**Nitrate data:** a chronic dose of 260 mg kg b.w. nitrate per day (equivalent to 8,650 mg/kg feed on dry matter), had no immediate effect on sheep but deaths occurred 2-3 months later (Setchell and Williams 1968).

**6.2. Pigs**

Pigs under intensive farming conditions are usually fed on complete feeds based on cereals and grains which have very low natural nitrite levels and this normally obviates adverse effects from feed and water can be the greater problem. Pigs are also amongst the most sensitive species due to their low methaemoglobin reductase activities in red blood cells (Kuehnert, 1991).

**Nitrite data:** Muirhead and Alexander (1997) reported that a single oral dose of nitrite above 20 mg/kg b.w. was lethal to pigs. Wendt et al. (1985) described a lethal dose of 70 mg/kg b.w. Recent case reports on lethal nitrite intoxication in pigs through consumption of contaminated water with 78 g/L nitrite resulting in estimated lethal doses of 21 and 70 mg/kg nitrite b.w., for sows and weaning pigs respectively (Vyt et al., 2005; Vyt and Spruitte, 2006).
Experimentally, Koch et al (1963) reported an increased formation of methaemoglobin in pigs fed a diet containing 150 mg/kg nitrite b.w. per day. A level of 100 mg/L nitrite in water, corresponding to approximately 10 mg/kg b.w. per day was sufficient to increase significantly methaemoglobin levels in treated pigs, however no clinical signs were observed and only at 20% methaemoglobin do clinical signs become evident, starting with restlessness and dyspnoea, and ending in ataxia, vomiting and cyanosis (Seerley et al., 1965). This value of 10 mg/kg b.w. per day can be considered as the LOAEL for pigs. No teratogenic effects were observed in foetuses examined 10 days after sows were exposed to a treatment with 17.2 mg/kg b.w. nitrite, on days 15, 31, 32 and 33 of gestation (Sleight et al., 1972).

Nitrate: methaemoglobinemia has also been described in pigs as a result of nitrite intake from the consumption of whey and the formation of nitrite from nitrate in a liquid cooked feed (Bruning-Fann and Kaneene, 1993).

6.3. Rabbits

Nitrite data: Dollahite and Rowe, (1974) experimentally induced acute toxicity in New Zealand White rabbits from an oral dose of 88 mg/kg b.w. per day nitrite for a 2.2 kg rabbit, fed on 0.13 kg DM per day. Violante et al. (1973) reported changes in urinary steroid excretion of rabbits as a time-dependent decrease in the urinary excretion of 17-hydroxy-, 17-keto- and 17-ketogenic steroid and caused by oral administration of 13.4 mg kg b.w. per day nitrite for 14 days. However, no methaemoglobinemia was noted.

6.4. Poultry

In poultry mostly fed seed-based grain, nitrite intoxication is rare, and is mostly caused by a contaminated water supply.

Nitrite data: Experimentally, a reduction in growth rate, an increase in the thyroid volume, and a higher death rate were seen in chickens fed a diet containing nitrite at dose of 75 mg/kg b.w. per day (Sells and Robert, 1963). Vitamin A supplementation resulted in the reduction of such effects. Atef et al. (1991) reported bio-chemical evidence of altered liver and kidney function along with a compromised reactivity of the immune system in chicks fed 1,122 mg/kg DM nitrite and corresponding to 80 mg/kg b.w. per day for a 2.1 kg broiler fed on a 0.15 kg feed.

6.5. Fish

In fish, the intestinal route accounts for about two-thirds of the whole body nitrite uptake (Grosell and Jensen, 2000); the remaining part is absorbed through the gills. No experimental data were available on exposure to nitrite via feeds; however, large amounts of unconsumed feed may result in high nitrite concentrations in water, as a result of the breakdown and fermentation. Factors that affect the nitrification process include pH, temperature, concentration of dissolved oxygen, number of nitrifying bacteria (such as those belonging to the Nitrobacter family), and the presence of inhibiting compounds, such as antibiotics. The formation of methaemoglobin results in the blood of the fish turning from red to a dull brown; this can be seen when examining the gills. Nitrite intoxication outbreaks have been described in fish (i.e. catfish) farmed in ponds or in aquaculture with water recycling facilities (Svoboda et al., 2005). Due to the competitive uptake with chloride, nitrite is generally less toxic in seawater than in freshwater, where salmonids have been found to be the most sensitive species (Bath and Eddy, 1980). Fish with high branchial chloride uptake rates (e.g. rainbow trout, perch, pike) were also shown to be more sensitive to nitrite than fish with low
uptake rates (eel, carp, tench) (Williams and Eddy, 1986). Other factors influencing the nitrite toxicity in fish is the oxygen concentration in water and the pH; oxygen concentration above 5 ppm in water could mitigate the hypoxia, while pH values below 6 may enhance the nitrite toxicity. For carp, 80 mg/L is considered to be the maximum admissible nitrate concentration in surface water, while 20 mg/L represent the threshold level for rainbow trout (Svoboda et al., 2005). For nitrite, in farmed tilapia the median lethal concentration (LC$_{50}$) of nitrite at 96h was 28.18 mg/L in water with low chloride content (35.0 mg/L) and 44.67 mg/L with high chloride content (70.0 mg/L, respectively) (Yanbo et al., 2006). In farmed trout, EFSA (2008b) recommended that nitrite levels in water should be below 0.1 mg/L.

6.6. Horses

Horses do not convert nitrate to nitrite efficiently and, are thus are less susceptible to poisoning compared with ruminants. No specific data on nitrite toxicity are available. Nitrate: methaemoglobinemia has been induced in two horses after a single dose administration of 50 g as nitrate per horse, approximately equivalent to 100 mg/kg nitrate b.w. per day and 3,300 mg/kg DM for a 500 kg b.w. animal, with a 15 kg DM daily intake. The onset of the signs appeared within 11-21 hours from the intake. The dose of 100 g resulted in lethality, and was associated with a methaemoglobin of 70 % (Bradley, 1940). Horses are not ruminants and therefore they have less opportunity to further convert nitrite to safer forms such as ammonia.

6.7. Fur animals

No specific data on nitrite and nitrate toxicity are available in fur animals. Intoxication in mink and foxes due to the intake of fermented/degraded fish meals, has been extensively reviewed (Bruning-Fann and Kaneene, 1993). This occurred in former times due to the formation of nitrosamines in fish meal induced by the addition of sodium nitrite as a preservative (Bruning-Fann and Kaneene, 1993). The production of nitrosamines was encouraged by the acidic pH of the meal, that allowed the formation of N-nitroso compounds with NDMA as major product. This occurs, through the reaction of nitrite with naturally occurring secondary and tertiary amines, nitrogen oxides and quaternary nitrogen compounds. The toxicity observed was not related to methaemoglobinemia formation, but to a direct liver toxicity of dimethylnitrosamine. The first symptoms were restlessness, fur dryness, then, progressive inappetence and ascites, leading animals to death (Koppang, 1966).

6.8. Cats and dogs

Nitrite toxicity: Michalsky (1963) reported methaemoglobin induction in dogs after oral exposure to nitrite at doses ranging from 7.9 – 19.8 mg/kg b.w. per day with a minimum lethal dose of 40 mg/kg b.w. per day.

Nitrate data: a NOAEL of 370 mg/ kg b.w. of nitrate has been established from a subchronic toxicity study in dogs (Lehman, 1958; Walker, 1990).

In cats, acute toxicosis with death due to extensive methaemoglobin formation has been reported in three animals fed on a canned feed containing on average 2,850 mg /kg nitrite (Worth et al., 1997). No effects on the growth rate or on the weight of important organs were noted in one cat receiving 105 mg/kg b.w. of sodium nitrite (69 mg/kg b.w. as nitrite) during a period of 105 days (approximately 390 mg/kg in the daily diet) (JEFCA, 1974).
The literature reviewed above indicates that with the acknowledged limitations, the values reported in Table 12 may be considered as reasonable approximations of LOAELs and NOAELs for a range of livestock and companion animals.

Table 12. LOAELs and estimated NOAELs derived from the lowest exposure to nitrite (or nitrate) reported to induce toxicity in livestock and companion species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>Toxic endpoint</th>
<th>LOAEL mg/kg b.w. per day</th>
<th>NOAEL(^1) mg/kg b.w. per day</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle*</td>
<td>Nitrate in feed (estimated to nitrite)</td>
<td>Not stated (MHb)</td>
<td>9.9</td>
<td>3.3(^2)</td>
<td>No data for nitrite, extrapolated from nitrate using factor of 10, and from LD(_{50}) using a factor of 10</td>
</tr>
<tr>
<td>Calves</td>
<td>Nitrite per se</td>
<td>MHb</td>
<td>34</td>
<td>11</td>
<td>Baranova et al., (2000)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Nitrite per se</td>
<td>Not stated (MHb)</td>
<td>10</td>
<td></td>
<td>Trif et al., (1993)</td>
</tr>
<tr>
<td>Growing Pigs</td>
<td>Nitrite in feed</td>
<td>MHb</td>
<td>10</td>
<td>3.3</td>
<td>Koch et al., (1963)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Seerley et al., (1965)</td>
</tr>
<tr>
<td>Sows</td>
<td>Nitrite per se</td>
<td>Lack of developmental defects</td>
<td>17.2</td>
<td></td>
<td>Sleight et al., (1972)</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Nitrite per se</td>
<td>Urinary hormone excretion changes</td>
<td>13.4</td>
<td>4.5</td>
<td>Violante et al., 1974</td>
</tr>
<tr>
<td>Poultry</td>
<td>Nitrite per se</td>
<td>Liver and kidney function</td>
<td>75</td>
<td>25</td>
<td>Sells and Roberts, 1963</td>
</tr>
<tr>
<td>Horses*</td>
<td>Nitrate in feed (estimated to nitrite)</td>
<td>MHb</td>
<td>10</td>
<td>3.3</td>
<td>No data for nitrite, extrapolated from nitrate using factor of 10</td>
</tr>
<tr>
<td>Cats</td>
<td>Nitrite in food</td>
<td>MHb</td>
<td>69</td>
<td>23</td>
<td>JEFCA, 1974</td>
</tr>
<tr>
<td>Dogs</td>
<td>Nitrite per se</td>
<td>MHb</td>
<td>7.9</td>
<td>2.6</td>
<td>Michalsky, 1963</td>
</tr>
<tr>
<td>Fish (trout)**</td>
<td>Nitrite per se</td>
<td>MHb</td>
<td>0.1(^3)</td>
<td></td>
<td>EFSA, 2008b</td>
</tr>
</tbody>
</table>

* Estimated from feed exposure to nitrate using a 10:1 ratio for nitrite:nitrate ratio- **Data referred to one animal, ***Data reported on water, \(^1\) A safety factor of 3 is applied to the LOAEL to derive the NOAEL value, \(^2\) based on nitrate LD\(_{50}\), \(^3\) mg/L, MHb; methaemoglobinemia,

The adverse effects of nitrite as demonstrated by the LOAELs derived in different livestock and companion animals (Table 12) broadly lie in the same range allowing for the different species sensitivities already discussed. Interpretation of the data is restricted as some findings relate to bolus administration of the nitrite or nitrate per se, whereas other studies relate to the nitrite and or nitrate resulting from that contained within the feedingstuff. The latter scenario generally results in less toxicity and is more akin to the normal feeding and drinking habits of farmed livestock. However, the consequence of bolus dosing could be considered as the most conservative scenario. Of the food producing livestock, cattle and pigs are known to be relatively sensitive to nitrite. This is at least partially explained by physiological factors, *vide supra* and confirmed by the figures estimated as NOAELS shown in Table 12.

7. Toxicokinetics

The toxicokinetics of nitrite together with a brief summary of nitrate is discussed in this section. For a more complete report, the reader is referred to the CONTAM Panel opinion on nitrate in vegetables (chapter 8.1) (EFSA, 2008a).
7.1. Absorption

The rate of absorption of nitrite and nitrate varies between species being relatively high in humans and rats but it is lower in ruminants and both are quantitatively well absorbed from the stomach and upper intestine/rumen.

For example, in humans, 90-95% of orally administered sodium nitrite is absorbed from the gastrointestinal tract and an average 25-fold increase in plasma nitrate was found 10 min after ingestion of nitrate (EFSA, 2008a). It has also been estimated that in humans approximately 25% of an ingested nitrate dose is secreted in the saliva (Bartholomew and Hill, 1984), and that from 20 to 46% of this, 25% is reduced to nitrite by oral micro-organisms (Spiegelhalder et al., 1976; Packer et al., 1989).

In dogs, large amounts of nitrate are secreted in saliva and bile (Fritsch et al., 1985). However in the rat, nitrate is mainly secreted in the gastric and intestinal fluid by entero-systemic recirculation (EFSA, 2008a).

Non-ruminants, such as horses and pigs, convert nitrate to nitrite in the intestine closer to the end of their digestive tracts with a minor absorption of nitrite (Yaremcio, 1991). In ruminants, only about 10-20% of nitrite and nitrate present in the rumen pass into the blood stream as nitrite, while the rest of the nitrite and nitrate is metabolized by the microorganisms to ammonia. The ammonia is utilized by rumen microflora or eliminated with other gases during eructation and the remainder is absorbed in considerable quantities directly into the blood (Lewis, 1951; Winter, 1962; Wang et al., 1961). Absorbed nitrate can also be recycled into the rumen via saliva (Schneider and Yeary, 1975; Yaremcio, 1991). Salivary, biliary, pancreatic or direct intestinal secretions of nitrate may also contribute to the pool of nitrate present in the small and large intestines (Witter et al., 1979a, 1979b).

When high levels of nitrate are consumed (e.g. from contaminated water), normal microbial conversion of nitrate to nitrite and assimilation post-absorption can be overwhelmed resulting in excessive nitrite absorption and resultant metHb formation.

7.2. Distribution

Once absorbed, nitrite is rapidly distributed in the plasma with rapid binding to the erythrocytes (Schneider and Yeary, 1975; Dejam et al., 2007). At excessive levels of absorption or nitrite formation, nitric oxide formation can also result in methaemoglobinemia.

Interspecies differences in the volume of distribution of nitrite have been found in the dog, sheep and pony with respective values of 1624, 278 and 192 ml/kg b.w., respectively after intravenous administration of 20 mg/kg sodium nitrite b.w. (Schneider and Yeary, 1975). In a recent study in twenty humans, 0.11 mg/kg/min nitrite over five 5 min was administered intravenously and a volume of distribution at steady state of 5.2 L and 25.2 L were determined (Dejam et al., 2007). In vitro studies investigated protein binding in canine plasma and the extent of nitrite-protein binding was dose related and ranged from 4.5 to 13.6% bound within 5 to 25 μg/min/ml sodium nitrite but not at higher concentrations (50 to 100 μg/min/ml sodium nitrite 9.8 to 8.8%). Less than 1% of nitrate was bound to canine plasma within the whole dose range (5 to 100 μg/min/ml) (Schneider and Yeary, 1975).

The volumes of distribution of nitrate reported for humans, ponies, sheep and goats are similar and amount to 210-330 ml/kg b.w. (Schneider and Yeary, 1975; Schultz et al., 1985, Lewicki et al., 1998; EFSA, 2008a).
7.3.  Metabolism

After binding to plasma and erythrocytes, nitrite, when present at normal levels is metabolised to nitrate in animals which is then metabolised to nitric oxide, which has a wide range of physiological functions. After transport to the stomach, the acidic conditions will rapidly transform nitrite to nitrous acid, which in turn spontaneously decomposes to nitrogen oxides including nitric oxide. Exogenous intake of nitrite/nitrate leads to nitric oxide in the upper intestine. These levels are up to 10,000 times higher compared with levels resulting from endogenous production of nitric oxide from L-arginine by nitric oxide synthases (McKnight et al., 1997). In parallel, endogenous nitrate synthesis occurs through the L-arginine-NO synthase pathway. Endogenous conversion from nitrate also occurs in humans and is approximately 1 mg/kg b.w. per day for a 70 kg adult (EFSA, 2008a).

However with excessive uptake, nitrite can react with ferrous deoxyhaemoglobin (HbFe\(^{2+}\)) in mammalian erythrocytes to generate methaemoglobin and nitric oxide (HbFe\(^{3+}\)) and iron-nitrosyl-haemoglobin (HbFe\(^{2+}\)-NO) leading to the observed methaemoglobinaemia in animals and humans (Schneider and Yeary, 1975; Dejam et al., 2007; Jensen, 2003). Additionally, an autocatalytic oxyhaemoglobin-nitrite reaction necessary to oxidize iron-nitrosylhaemoglobin and release free NO occurs (Gladwin et al., 2009).

In the case of humans, dogs and mini-pigs, nitrate is concentrated from the plasma to the saliva and then the commensal bacteria present on the back tongue reduce approximately 20 % of the secreted nitrate to nitrite, which is then swallowed into the stomach. Nitrate is also secreted in the gut (EFSA, 2008a).

Non-ruminants, such as horses and pigs, convert nitrate to nitrite in the intestine closer to the end of their digestive tracts, reducing absorption into the blood and reducing likelihood of intoxications. However, these species are highly susceptible to oral nitrite intake (for example, in mouldy hay) because they cannot convert from nitrite to ammonia (Yaremco, 1991). The significant role of alimentary tract micro-organisms in the metabolism of nitrate has been demonstrated in experiments with germ-free (monogastric) rats which excreted in urine approximately 20 % more nitrate than conventional rats (Schultz et al., 1985).

In ruminants, nitrate is reduced to nitrite and ammonia by forestomach microorganisms that possess nitrate reductase activity (Lewicki et al., 1998, Witter et al., 1979a, 1979b, Hartman, 1982). However, heavy dietary loads of nitrate (higher than 65 mmol/rumen fluid) may overwhelm the ability of rumen microorganisms to completely degrade nitrate to ammonia and levels of nitrite, and intermediate compounds, may increase (Baranova et al., 2003). Nitrite is toxic to cellulolytic bacteria (Marais et al., 1988) and this can result in reduced digestibility of fibre in the rumen, which, in turn, will result in reduced feed intake and milk production. However, the ability of micro-organisms to completely break down nitrate to ammonia increases with the nitrate load. If nitrite production is so high that rumen microorganisms, breaking it down to ammonia, are overwhelmed, it will accumulate in the rumen, pass through the rumen wall to the blood and result in methaemoglobin formation (Robinson, 1999).

7.4.  Excretion

Nitrite is rapidly and extensively excreted in urine and thus does not accumulate in tissues. However, nitrate has a slower rate of excretion and the major part of the primary urinary nitrate (ca 80 %) is pumped back to the blood by an active transport mechanism. This salvaging of nitrate from the urine, in addition to the known recycling of nitrate from saliva
and also from the intestines (after biliary excretion) further suggests that the body is acting to conserve a substance of physiological importance (EFSA, 2008a).

In humans, mean clearance and inter-compartmental clearance for nitrite using a one compartment model were 0.948 and 0.67 L/min respectively, with a terminal half-life of 42 minutes (Dejam et al., 2007).

Elimination kinetics of nitrite in the dog, sheep and pony were determined after intravenous administration of 20 mg sodium nitrite/kg b.w. and the elimination half-lives of nitrite were approximately 0.5 hours in the dog, sheep and pony (Schneider and Yeary, 1975). However, the clearance of nitrite in the dog was higher (38 ml/min/kg) than that in the pony (3.8 ml/min/kg) and in the sheep (4.1 ml/min/kg). The large clearance values for nitrite in the dog indicate that some process other than renal excretion, such as metabolic conversion of nitrite to nitrate by erythrocytes, contributed significantly. It has been reported that 80-90% of the amounts of nitrate orally and intravenously administered to dogs as sodium nitrite were recovered in the urine (Greene and Hiatt, 1954). Thus, significant tubular reabsorption of nitrate in the dog probably occurs, as shown by the slow rate of elimination observed (Schneider and Yeary, 1975).

After a single oral administration of 60 mg/kg b.w. of aqueous sodium nitrite to calves on milk nutrition, the highest nitrite 7.8 mg/L and nitrate 289 mg/L values in urine were determined after 2 h. After the administration of 480 mg/kg b.w. of potassium nitrate, a mean value of 2540 mg/L nitrate was measured after 3 h. These results clearly demonstrate a good trans-renal passage of nitrite and nitrate in calves on milk nutrition (Baranova et al., 2000).

8. Carry-over and residues

No trials have been located which describe the transfer of nitrite into animal products following normal feeding. However, the transfer of nitrates into the milk of dairy cows following nitrate loading with potassium nitrate at 2-week intervals as single oral doses of 9.5, 18.75, 37.5, 75 and 150 g two hours before evening milking has been investigated. Nitrate residues were quantified in individual milk samples obtained from manual milking at time intervals of 2, 14, 26, 38 and 50 hours after application of potassium nitrate. Average values for nitrate residues in milk two hours after administration were low with 3.4, 4.5, 9.8, 15.6 and 34.6 mg of nitrate/L respectively (Baranova et al., 1993).

Eleftheriadou et al. (2002) analysed nitrite and nitrate levels from 120 muscle samples from 5 slaughtered pig breeds. In addition, 20 feed samples and 20 water samples were analysed. Only trace amounts of nitrite, and low concentrations of nitrate were found in the samples (7.5-15.7 mg nitrate/kg in meat, 9.3 to 13.4 mg nitrate/kg in animal feed and from 28.0 to 65.2 mg nitrate/L in the water). However, there was no correlation between the nitrate quantity in feed and water and its concentration in meat.

Overall, data on the carry-over and residues of nitrite in animal tissues and animal-based products are very scarce. However, because of the rapid excretion of nitrite and nitrate, the likelihood of accumulation in animal tissues and animal-based products is considered to be low.

9. Animal risk assessment

Based on published adverse effect data, NOAELs for nitrite have been estimated for monogastric and ruminant livestock (table 12). Physiologically, pigs and adult cattle represent sensitive livestock species with regard to nitrite exposure and NOAELs of 3.3 mg/kg b.w. per
day were estimated for both species (table 12). The nitrite intake for both species was also estimated using the maximum exposure level in complete feed based on the current legislation (10 mg/kg) and typical feeding regimens feed within Europe (table 8 and 9). In addition, the maximum level in forages from member states (Slovenia (26.2 mg/kg) was also taken into account to calculate exposure in cattle (table 9). Overall, nitrite intake from feed was 0.37 and 0.65 mg/kg b.w. per day in pigs and cattle which compared with the estimated NOAEL of 3.3 mg/kg b.w. per day gives margins of safety of 9 and 5 respectively. Overall, allowing for farmer awareness of the risk of nitrite poisoning in livestock from feed intake, these margins of safety over typical dietary intake are considered adequate to protect animal health.

Other important factors that farmers consider for good agricultural practices are the naturally high levels of nitrate in forages, silages, hays and in potentially contaminated water (wells, surface water contaminated with bacteria). Such high levels can potentially affect susceptible livestock groups on an acute basis and potentially on a cumulative basis, particularly in ruminants (pregnant cows, calves) because of the rumen conversion of nitrate to nitrite. However in the case of cattle, rumen adaptation plays a key role in the prevention of such adverse effects and dietary nitrite from feed is not considered to be a practical risk for animal health.

10. Human intoxication

Nitrite causes methaemoglobinaemia, usually as a consequence of excessive nitrate exposure. While cases of methaemoglobinaemia have been widely reported particularly in infants, specific data relating to direct poisoning by nitrite per se are scarce. A case of human intoxication to nitrite in a 40 year-old mother and her child (9 years) was however recently described after the consumption of turkey contaminated with nitrite levels ranging from 6,000 to 10,000 mg/kg. This resulted in high levels of methaemoglobinaemia in the mother (32.7 %) and the child (62.3 %), 2 hours after consumption of the turkey. Five hours later, methaemoglobinaemia was back to normal levels in the mother (1.6 %) whereas in the child methaemoglobin levels were still above 20 % but returned to normal within 12 hours (2.3 %) (Matteucci et al., 2008).

Five cases of methaemoglobinaemia (21 %-57 %) after ingestion of sodium nitrite occurred in Sydney in 2006. All cases were unintentional poisonings. Blood levels of nitrite were not reported (Maric et al., 2008).

Intoxication of infants below 6 months due to high nitrate exposure are widely reported in the literature. Infants, particularly under 3 months of age, have a much greater susceptibility to metHb compared with adults due to a 40-50 % lower level of activity of NADH-cytochrome b5 methaemoglobin reductase which is responsible for converting metHb back to Hb. Additionally, gastro-enteritis can be another factor resulting in increased bacterial conversion of nitrate to nitrite (EFSA, 2008a). A case reported methaemoglobin levels of 27 % and 30.4 % in two infants from Italy, aged 1 and 2 months respectively, fed with a formula reconstituted with a high concentration of courgette soup to resolve constipation. Both were hospitalized after developing severe cyanosis and were treated with methylene blue at 1 %: after 12 h the syndrome was completely resolved (Savino et al., 2006). Another study carried out in Spain assessed the causes of methaemoglobinemia in seven infants with levels ranging from 10 % to 58 % and in all cases these were associated to nitrituria in relation to consumption of mixed vegetables and incorrect storage of homemade purees. In this case, the highest levels of nitrite-forming nitrate were recorded in silver beets with 3200 mg/kg of the vegetable (Sanchez-Echaniz et al., 2006).
11. Human dietary exposure and risk assessment

Contributors to exogenous dietary nitrite intake in humans are vegetables and fruits, water, animal-based products, and other foods approximating a total dietary intake of between 1.5 and 2.0 mg/person/day (EFSA, 2008a). Using the UK dietary study, the contribution from non-preserved animal products (e.g. milk, fresh meat and eggs) is approximately 14 % of the total exogenous nitrite intake. However, the figures for exogenous nitrite intake are dwarfed by the nitrite endogenously generated from the dietary consumption of nitrate which by far represents the largest contributor (>80 %) to human nitrite pools resulting in a total combined exposure of some 7.3 mg nitrite/person/day.

Nitrite is also formed to a limited extent endogenously in different tissues and further oxidation can result in the production of nitrate (Lundberg et al., 2004 and 2008; EFSA, 2008a).

Nitrite levels have not been reported in milk. Average concentrations of nitrate in dairy products were 27 mg/kg and in milk ranged from 3.9–5.3 mg/kg from the UK total diet study (MAFF, 1998). Other studies consistently reported nitrate levels in cows’ milk and cheese, without nitrate additives, below 5 mg/L and 1–8 mg/kg respectively (MAFF, 1998; Walker et al., 1990; Kammerer et al. 1992; Gangolli et al., 1994).

Nitrite levels have not been found in detectable amounts in ten egg samples (Ologhobo et al., 1996) and the average concentration of nitrate in eggs (4.4-5.4 mg/kg) was estimated in the United Kingdom total diet study to range from (range, undetected–12 mg/kg) (MAFF, 1998).

Concentrations of nitrite and nitrate in cured meat were 4.1 mg/kg (range, 1.5–8.4 mg/kg) and 45 mg/kg (range, 14–101 mg/kg) respectively. More specifically, nitrate levels in bacon, and other cured meat ranged from 1.4 to 440 mg/kg and from 0.2 to 450 mg/kg respectively (MAFF, 1998). In the USA, Cassens et al. (1997) found residual nitrite in 164 samples of cured meat in three trials showing average concentrations of 5–10 mg/kg (range, 0–48 mg/kg). Sausages (e.g. hot dogs) had a mean content of about 100 mg/kg nitrite and fried bacon and fried ham had about 35 mg/kg nitrite (National Academy of Sciences, 1981).

Total daily dietary exposure for both nitrate and nitrite were estimated and expressed as a percentage of the total diet, for the UK as an example of a Northern European country (MAFF, 1998) and France as an example of a Central/Southern European country (modified after Causeret, 1984). For both countries the largest source of nitrite is endogenous conversion from nitrate.

In the UK, nitrite dietary exposure, estimated by means of a total diet study, represents on average 1.5 mg per day per person. Fresh meat represents only 4 % of total intake, which along with other fresh animal products, milk and dairy products (6 %), eggs (3 %) and fish (1 %) combines to a total of 14 %. Other contributors include preserved meat products (19 %), vegetables and fruit (15 %), water (7 %). The remaining 47 % comes from other foods such as bread, cereals, oil and fats, sugar preserves, beverages and nuts (MAFF, 1998). Nitrate intake is much larger (91 mg per day per person) and is mostly through the consumption of vegetables (52 %), water (22 %), beer (12 %), other foods (6 %). Fresh meat, meat products, milk and dairy products, eggs only represent a minor fraction of nitrate exposure with respective values of 1 %, 3 %, 3 %, and 0.2 %. Overall the total nitrite exposure that includes endogenous conversion from nitrate exposure in the UK using a conservative factor of 7 % for the ingested conversion nitrate to nitrite was approximately 8.8 mg per person per day (EFSA, 2008a). In comparison to nitrite intake, bioconversion of nitrate to nitrite represents some 82 % of the total nitrite intake.
From the French study, nitrite average dietary exposure represents 2 mg per person per day and is mainly through vegetables and fruit (41 %), animal-based products (39 %), water (14 %) and other food (4 %). Nitrate intake is 141 mg per day per person and mostly through the consumption of vegetables (75 %), water (14 %), animal-based products (4 %), beer (1 %), other foods (4 %). The conversion of nitrate to nitrite was approximately 9.3 mg per person per day representing 85 % of the total nitrite intake (11.3 mg per person per day) (EFSA, 2008a).

From these figures, the major source of human exposure to nitrite is the inter-conversion from nitrate in vegetables, cured meat products and water intake, which represents over 80 % of the total daily exposure. By contrast, direct exogenous nitrite intake from the diet constitutes less than 20 % with the major contributors being vegetables, fruits, water and other foods. Overall, levels in fresh animal-based products, fish, milk dairy products, and eggs only represent approximately 2.9 % of the total combined daily nitrite exposure. Such a small proportion of the total daily intake to nitrite does not raise any concern for human health.

**CONCLUSIONS AND RECOMMENDATIONS**

**CONCLUSIONS**

*Chemistry, occurrence in plants and feed materials*

- Nitrite is formed naturally by the nitrogen cycle during the process of nitrogen fixation. It is subsequently converted to nitrate, a major nutrient assimilated by plants.
- Animal feed can contain nitrite from its natural plant constituents (generally very low levels), from microbial action in moist feeds/stored plant materials e.g. hay and silage, or when nitrite is added directly as a preservative. Nevertheless, the majority of nitrite exposure in livestock results from the endogenous inter-conversion of nitrate in feed and water into nitrite.
- Current European Union (EU) legislation for nitrite, Annex 1 of Council Directive 2002/32/EC, sets maximum limits for nitrite in complete feed for livestock of 15 mg/kg sodium nitrite (10 mg/kg nitrite at 12 % moisture) and 60 mg/kg in fish meal sodium nitrite (40 mg/kg nitrite). Under normal manufacturing/husbandry practice the concentration of nitrite in the majority of these feeds is likely to be well below these figures as confirmed by limited evidence from 3 Member States.
- No official analytical method for the determination of nitrite in animal feed is prescribed by EU legislation nor established by the European Committee of Standardization (CEN).

*General toxicological effects*

- The acute toxicity of nitrite is approximately 10-fold higher than that of nitrate. The main adverse effect related to acute toxicity of nitrite is methaemoglobinaemia occurring in both animals and man.
- Long-term toxicity of nitrite is associated with a dose-related trend (not statistically significant) for squamous cell papilloma or carcinoma in the forestomach of female mice with hyperplasia of the epithelium of the glandular stomach at the highest dose level in males.
The acceptable daily intake (ADI) value for nitrite is 0-0.07 mg/kg body weight (b.w.) per day as established by the Joint FAO/WHO Expert Committee on Food Additives and endorsed in the recent risk benefit assessment of nitrate in vegetables by the Panel on Contaminants in the Food Chain (CONTAM Panel).

**Adverse effects of nitrite on livestock**

- Nitrite poisoning in livestock generally only occurs due to metabolic overload in the presence of contaminated feed or water and is usually due to the presence of excessive nitrite inter-converted from nitrate. This can result from fermented silage or water polluted by animal waste, particularly in the presence of bacterial contamination. In animals, nitrate from feed and water are normally reduced to nitrite and converted to ammonia for anabolism to amino acids. This is an adaptive process.
- The adverse effects of nitrite in animals are dominated by methaemoglobin formation. At low exposure levels methaemoglobin is reversed by a nicotinamide adenine dinucleotide-dependent reductase, the activity of which shows large species and age dependent variations.
- Sensitive species of food producing animals include the pig, which has relatively low reductase activity in comparison with other species and cattle which are particularly susceptible due to their physiology enabling rapid rumen conversion of high levels of exogenous nitrate (e.g. from water pollution) to nitrite. No observable adverse effect levels (NOAELs) for pigs and cattle have been estimated from the literature with values of 3.3 mg/kg b.w. per day for both species.

**Fate in animals and carry-over**

- Due to the rapid metabolic turnover and excretion of nitrite and nitrate, the potential for accumulation in animal tissues is low. Residues of nitrate were found in muscle samples from 5 pig breeds ranging from 7.5 to 15.7 mg/kg. There was no correlation between the nitrate concentration in feed and water and its concentration in meat.
- Overall, there is very little quantitative information available on nitrite residues in animal products. However, any residues of nitrite in animal products are likely to be very low, compared to those arising from fruit and vegetables via the inter-conversion of nitrite.

**Relevance to animal health**

- Total nitrite intake for pigs and cattle from feed as representative sensitive food producing species was estimated using the maximum exposure level in complete feed from the current legislation (10 mg/kg) and typical feeding regimens within the EU. For cattle, the maximum nitrite level in forages from member states was also taken into account (Slovenia, 26.2 mg/kg). Overall, nitrite intake per se was 0.37 and 0.65 mg/kg b.w. per day for both pigs and cattle corresponding to margins of safety of 9 and 5 with respect to the respective NOAEL. The CONTAM Panel considered that such margins do not pose concerns for animal health given farmer awareness of nitrite poisoning and good agricultural practice.
Human exposure

- Human dietary exposure to nitrite is mostly through the inter-conversion of nitrate from vegetables, cured meat products, water intake which represents over 80 %, whereas exogenous nitrite intake from the diet constitutes less than 20 %. Overall, the levels in fresh meat, fish, milk dairy products, and eggs, only represent 2.9 % of the total combined daily nitrite exposure in man. Therefore, the CONTAM Panel concluded that such a contribution of nitrite from animal products does not raise any concern for human health.

Recommendation

Since the analytical methods measure the nitrite ion, it would be helpful to base any future legislation on the nitrite ion and not on sodium nitrite.
The EFSA Journal (2009) 1017, 36-47

REFERENCES


Nitrite as undesirable substances in animal feed


Nitrite as undesirable substances in animal feed


Nitrite as undesirable substances in animal feed


Nitrite as undesirable substances in animal feed


NIOSH (The National Institute for Occupational Safety and Health, USA), 1987. NIOSH Registry of toxic effects of chemical substances.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ADI</td>
<td>Acceptable Daily Intake</td>
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<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
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<td>b.w.</td>
<td>body weight</td>
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<td>CEN</td>
<td>European Committee for Standardisation</td>
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<td>CFU</td>
<td>colony-forming unit</td>
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<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<td>Panel on Contaminants in the Food Chain</td>
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<td>CVMP</td>
<td>Committee for Medicinal Products for Veterinary Use</td>
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<td>European Food Safety Authority</td>
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<td>FAO/WHO</td>
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<td>Good Agricultural Practice</td>
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<tr>
<td>LD₅₀</td>
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<tr>
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<td>steroid acute response protein</td>
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