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Revision of the currently authorised maximum copper content in complete feed

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

Abstract

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) reviewed (i) the copper requirements of food-producing and pet animals, (ii) the copper concentration in feed materials and complete feed, (iii) the copper bioavailability, and (iv) the calculated background copper concentration of complete feed. Also considered were (i) the influence of dietary copper on gut microbiota profile and on the bacterial antibiotic resistance in target animals and (ii) the environmental occurrence of bacterial heavy metal tolerance (copper resistance) and resistance to certain antibiotics. The data collected supported the possibility of a reduction in some of the currently authorised maximum contents (CAMC) for total copper in feed. The EFSA Panel developed an algorithm to derive newly proposed maximum contents (NPMC) from the requirement and the native dietary copper content. The NPMC (mg Cu/kg complete feed) comprised of maintained (m), decreased (d) and increased (i) values: 15 for bovine before the start of rumination (m), 30 for other bovine (d), 35 for caprine (i), 15 for ovine (m), 50 for crustacean (m) and 25 for other animal species ((d) for piglets up to 12 weeks, (m) for all other species). The NMPC support health, welfare and economic productivity of target animals, except piglets; performance of weaned piglets would be impacted. The NPMC values would not likely have any consequences on the consumers' intake of copper and are of no concern for the safety of the consumer. The reduction from 170 mg to 25 mg Cu/kg feed piglets would have the capacity to save 1,200 tonnes copper/year being spread in the field and thus, to reduce total copper emissions from farm animal production by about 20%. Thus, the reduction of the CAMC to the NPMC would have a significant impact on the concentrations of copper in the environment of piggeries.

Keywords: copper requirements, copper in feed, interactions/incompatibilities, gut microbiota, antimicrobial resistance, maximum copper content in feed, safety

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Correspondence: feedap@efsa.europa.eu

Panel members: Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Secundino Lopez Puente, Marta Lopez-Alonso, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester

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Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the revision of the currently authorised maximum copper content (CAMC) in complete feed (in mg Cu/kg complete feed; 170 for piglets up to 12 weeks, 25 for other pigs, 15 for bovine before the start of rumination, 35 for other bovine, 15 for ovine, 25 for fish, 50 for crustaceans, 25 for other animal species).

To improve the available information on the use of copper in animal nutrition, the European Food Safety Authority (EFSA) launched a call for data to European Union (EU) Member States (MS) and EEA/EFTA countries and to stakeholders. EFSA awarded procurements on an Update of the Systematic Literature Review (SLR): 'Influence of Copper on antibiotic resistance of gut microbiota on pigs (including piglets)' and on an Extensive Literature Search (ELS) on the 'Effects of Copper intake levels in the gut microbiota profile of target animals, in particular piglets'. The data submitted were used in the current scientific opinion.

The Panel reviewed (i) the copper requirements of food-producing and pet animals, (ii) the copper concentration in feed materials and complete feed, (iii) the copper bioavailability, and (iv) the calculated background copper concentration of complete feed. Also considered were (i) the influence of dietary copper on gut microbiota profile and on the bacterial antibiotic resistance in target animals and (ii) the environmental occurrence of bacterial heavy metal tolerance (copper resistance) and resistance to certain antibiotics. The data collected supported the possibility of a reduction in some of the CAMC for total copper in feed.

The Panel developed an algorithm to derive newly proposed maximum contents (NPMC) from the requirement and the native dietary copper content. The NPMC (mg Cu/kg complete feed) comprised of maintained (m), decreased (d) and increased (i) values, and were: 15 for bovine before the start of rumination (m), 30 for other bovine (d), 35 for caprine (i), 15 for ovine (m), 50 for crustacean (m) and 25 for other animal species ((d) for piglets up to 12 weeks, (m) for all other species).

The NMPC support health, welfare and economic productivity of target animals, except piglets; the performance of a weaned piglet would be reduced.

The reduction of the CAMC for food-producing animals to the NPMC values would not be likely to have any consequences on the consumers' intake of copper and is of no concern for the safety of the consumer.

The reduction of copper in feed for piglets from 170 mg/kg to 25 mg/kg would have the capacity to save 1,200 tonnes copper being spread in the field per year; the introduction of this NPMC could result in an overall reduction of about 20% of copper emissions from farm animal production. Thus locally, where manure from piglets is spread on land, the reduction of the CAMC to the NPMC would have a significant impact on the concentrations of copper in the environment of piggeries.

The FEEDAP Panel made some recommendations: (i) to keep the 'Other provisions' concerning molybdenum as in the current regulation; (ii) to initialise copper monitoring in the liver of slaughtered bovines; and (iii) to implement monitoring of copper pollution from agriculture in areas in which food-producing animals are fed, with particular attention to the potential development of microbial antibiotic resistance in the environment.

The FEEDAP Panel is of the opinion that MRLs for copper should not be set for tissues and products of animal origin, as also expressed by the EMA-CVMP. The use of copper is safely regulated by feed legislation. The authorised maximum copper contents in complete feed would effectively prevent any increase of copper in feed and, consequently, in food of animal origin.

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1. Introduction

1.1. Background and Terms of Reference

The opinions on applications for the re-authorisation of copper compounds have already been issued.[1] In the margins of the discussion with the Member States on those opinions, concerns with respect to the maximum contents of copper in feed have been raised.

The Commission asked the European Food Safety Authority (EFSA) to issue an opinion on the maximum content of compounds of copper in feed considered

- to fully meet the animals' nutritional needs for copper as a trace element, taking into account for the food-producing animals typical livestock farming systems, and
- to be safe for the animals, the consumer and the environment.

With the mandate, the European Commission (EC) provided a scientific paper on copper and antimicrobial resistance which had been already forwarded to the EFSA in 2011.[2] A statement from the Norwegian Food Safety Authority, including opinions from the National Institute of Nutrition and Seafood Research and the Norwegian Veterinary Institute, regarding the use of copper (also of zinc and iodine) in animal nutrition, was also provided to EFSA.[3]

A similar mandate had been forwarded in 2012 for zinc. The respective 'Scientific Opinion on the potential reduction of the currently authorised maximum zinc content in complete feed' was published in 2014.[4] The EC suggested that the methodology applied in the zinc opinion could serve as an example for the development of the opinion linked to the current copper mandate.

1.2. Interpretation of the Terms of Reference

In 2003, the Scientific Committee on Animal Nutrition (SCAN) was requested by the EC to deliver an opinion on the use of copper in feedingstuffs. The SCAN recommended that 'Current copper levels allowed in diets should be reviewed to better reflect animal requirements'; the following maximum copper contents were proposed: sheep, 15 mg/kg; pre-ruminant calves: 5 mg/kg milk replacer; piglets (up to 10 weeks of age), 175 mg/kg; other species/categories, 25 mg/kg.

Regulation (EC) No 1334/2003[5] set the following maximum total copper contents in feed (mg Cu/kg complete feedingstuff) (Table 1).

Table 1: Currently authorised maximum copper content in feed in the European Union

Animal species	mg Cu/kg complete feedingstuff
Pigs	
Piglets up to 12 weeks	170 (total)
Other pigs	25 (total)
Bovine	
1. Bovine before the start of the rumination:	
Milk replacers	15 (total)
Other complete feedingstuffs	15 (total)
2. Other bovine:	35 (total)
Ovine	15 (total)
Fish	25 (total)
Crustaceans	50 (total)
Other species	25 (total)

[1] Most recent one: EFSA Journal 2015;13(4):4057.

[2] Berg J, Thorsen MK, Holm PE, Jensen J, Nybroe O and Brandt KK, 2010. Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. Environmental Science and Technology, 44, 8724–8728.

[3] Letter dated 30.1.2015 submitted by the Norwegian Food Safety Agency to the European Commission concerning information on 'Copper supplementation of animal feed', including the report *Innhold av kobber og jod i fôr til fisk – Vurdering av endring av grenseverdi* [Copper and iodine in feed to fish] from NIFES (National Institute of Nutrition and Seafood Research).

[4] EFSA Journal 2014;12(5):3668.

[5] Commission Regulation (EC) No 1334/2003 of 25 July 2003 amending the conditions for authorisation of a number of additives in feedingstuffs belonging to the group of trace elements. OJ L 187, 26.7.2003, p. 11.

In its opinions on copper-containing additives, the EFSA FEEDAP Panel concluded that

> 'A reduction in the maximum copper content in feed for ruminants to bring it close to the minimum requirement would reduce copper concentration in the liver. However, this measure can hardly be realised in practice because of the varying occurrence of copper antagonists in feedingstuffs (mainly molybdenum and sulfur), particularly in roughages, requiring additional copper to prevent the risk of copper deficiency and its consequences on animal health'. (EFSA FEEDAP Panel, 2012)

> 'More recent findings on the copper requirements of animals indicate the potential to considerably reduce the current maximum content for dietary copper without affecting animal health and welfare and the productivity of animal husbandry. A reduction in the maximum content of copper would decrease the copper load in the environment'. (EFSA FEEDAP Panel, 2013)

The FEEDAP Panel considers that the request of the EC refers as to the issuing of an opinion on the revision of the currently authorised maximum copper content in complete feed, considering safety for animals, consumers and the environment.

1.3. Additional information

The SCAN delivered reports on the use of copper methionate for pigs (European Commission, 1981), copper compounds in feedingstuffs (European Commission, 1982) and in feedingstuffs for pigs (European Commission, 1983) and the use of copper in feedingstuffs (European Commission, 2003). EFSA has issued opinions on the safety of the chelated forms of iron, copper, manganese and zinc with synthetic feed grade glycine (EFSA FEEDAP Panel, 2005), on the safety and efficacy of a copper chelate of hydroxy analogue of methionine (Mintrex®Cu) as feed additive for all species (EFSA FEEDAP Panel, 2008, 2009), and on the safety and efficacy of dicopper chloride tri hydroxide (tribasic copper chloride, TBCC) (EFSA FEEDAP Panel, 2011) and dicopper oxide (EFSA FEEDAP Panel, 2016) as feed additives for all animal species. In the frame of re-evaluation, EFSA has delivered three opinions on copper-based additives: cupric sulphate pentahydrate (EFSA FEEDAP Panel, 2012), cupric chelate of amino acid hydrate (EFSA FEEDAP Panel, 2013) and seven compounds of copper (EFSA FEEDAP Panel, 2015).

Several compounds of copper are authorised for use in food, for the manufacturing of dietetic products, for the manufacturing of processed cereal foods and baby foods for infants and young children, as pharmacologically active substances, as fertilisers, as plant protection products and for cosmetic purposes (e.g. EFSA FEEDAP Panel, 2015).

EFSA delivered a Conclusion on the peer review of the pesticide risk assessment of confirmatory data submitted for the active substance copper (I), copper (II) variants namely copper hydroxide, copper oxychloride, tribasic copper sulfate, copper (I) oxide and Bordeaux mixture (EFSA, 2013).

2. Data and methodologies

2.1. Data

The FEEDAP Panel used data from previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, data provided by the mandate's requestor (the EC), and other scientific reports to deliver the present output. Moreover, the Panel used data from a specific data collection and a series of procurements, as described below.

EFSA launched a call for data to:

European Union Member States (EU MS) and European Economic Area/European Free Trade Association (EEA/EFTA) countries, concerning national copper requirements/allowances in animal nutrition, data on potential development of antibiotic resistance linked to the use of copper and data from official feed control for the copper content in compound feed. EFSA received contributions from Belgium, Cyprus, Czech Republic, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Montenegro, Norway, Poland, Portugal, Slovakia, Slovenia, Sweden, Switzerland and the United Kingdom.

Stakeholders, concerning recommendations for the use of copper in animal nutrition and typical composition of compound feed. Information was submitted by the European Pet Food Industry

Federation (FEDIAF), the European Feed Manufacturers' Federation (FEFAC) and the EU Association of Specialty Feed Ingredients and their Mixtures (FEFANA).[6]

EFSA commissioned the University of Gent (Belgium) and the University Rovira i Virgili (Spain) to carry out studies of selected trace and ultratrace elements, and on the bioavailability, interactions and incompatibilities of trace elements, respectively; the findings were submitted to EFSA in the form of technical reports (Van Paemel et al., 2010; Cano-Sancho et al., 2014). In the context of the current opinion, EFSA awarded two procurements on an Update of the Systematic Literature Review (SLR): 'Influence of Copper on antibiotic resistance of gut microbiota on pigs (including piglets)' and on an 'Extensive Literature Search on the Effects of Copper intake levels in the gut microbiota profile of target animals, in particular piglets', for which the reports of Van Noten et al. (2016) and Jensen (2016), respectively, were provided. Information from these reports has been used in this opinion.

2.2. Methodologies

The FEEDAP Panel applied the same methodology for the revision of the maximum copper authorised in feed, as it did in 2014 for the revision of the maximum content of zinc authorised in feed (EFSA FEEDAP Panel, 2014).[7] Namely, the Panel reviewed

1) the essentiality of copper,
2) the role of copper in animal nutrition,
3) the availability of copper and its interactions/incompatibilities with other dietary constituents,
4) the copper concentrations in feedingstuffs,
5) the influence of dietary copper on gut microbiota profile and bacterial antibiotic resistance in target animals and
6) the occurrence of bacterial heavy metal tolerance (copper resistance) and resistance to certain antibiotics.

The aim was to propose, where/if considered appropriate, new maximum copper levels in feed. Further to the newly proposed levels, the Panel evaluated their consequences on the health and welfare of target animals, on consumer supply and on the environmental load.

3. Assessment

3.1. Copper as essential trace element in nutrition

The information in this section and Annex A is derived from authoritative reviews and a recent EFSA Opinion (Stern et al., 2007; Suttle, 2010; Aggett, 2013; EFSA Scientific Panel on Dietetic Products, Nutrition and Allergies (EFSA NDA Panel), 2015; Ellingsen et al., 2015) in which specific references can be found.

Insights into the absorption, distribution, metabolism and excretion (ADME) of copper have been derived from studies in cell line systems *in vitro*, bacteria, yeasts, Drosophila, Xenopus, zebra fish, monogastric models (e.g. rodents and dogs), ruminants and humans. Knockout models and inborn errors of metabolism in mammals have contributed appreciably. The integration of information from these sources has been enabled appreciably by molecular biological techniques which have allowed the development of a mechanistically plausible model for the absorption and systemic fate of copper, as well as an indication of some differences in copper kinetics between species. Studies of these processes are hampered because the abundance of the two natural stable isotopes of copper, ^{63}Cu and ^{65}Cu of 69% and 31%, makes them difficult to use in tracer studies, and the use of copper's radio isotopes as tracers is limited by their short half-lives (^{67}Cu, 61.9 h and ^{64}Cu, 12.9 h). As a result, it is difficult to fully characterise the ADME of copper in individual species, and its intraspecies (e.g. sheep) and interspecies (monogastric and pre-ruminant animals and ruminant livestock) variation (EFSA NDA Panel, 2015; Ellingsen et al., 2015).

The essentiality of copper for all life forms, from bacteria to humans, depends on the ability of copper atoms to gain and lose an electron to form cuprous Cu(I) and cupric Cu(II) states. This

[6] To protect the interests of stakeholders that have contributed, they are collectively referred to in this scientific opinion as 'European feed industry'.

[7] This approach followed the proposal made by the requestor in the respective mandate: A similar mandate had been forwarded in 2012 for zinc. The respective 'Scientific Opinion on the potential reduction of the currently authorised maximum zinc content in complete feed' had been published. The methodology applied could serve as an example for this mandate.

transition is crucial for enzymes underpinning the metabolism of all major substrates (i.e. lipids, carbohydrates and proteins). It is therefore essential for, amongst other functions, mitochondrial respiration, energy production, the synthesis of collagen, neurotransmitters and catecholamines, in the uptake and utilisation of other trace metals, and in antioxidant activities.

Copper-dependent enzymes and their functions are listed in Annex A (Stern et al., 2007). Thus copper deficiency results, according to its severity, in protean features such as muscle weakness, iron-deficient anaemia, hypopigmentation, bone changes that resemble scurvy, defective connective tissue synthesis, hair abnormalities, impaired myelinisation of nerve tissues and neurological defects, altered lipid metabolism and cardiac malfunction (see Section 3.2.2).

The oxidation of cuprous to cupric copper generates free radicals which, in turn, can – by the oxidation of lipids, proteins, and nucleic acids – cause extensive architectural and functional damage in tissues and organs. Thus, organisms have developed systems involving specific protein carriers which mediate and regulate the absorption, distribution, use and excretion of copper whilst, simultaneously, controlling the production of free copper ions and minimising the risk of oxidative damage. Similar systems of chaperoned traffic exist for other elements such as iron, zinc and manganese. As these elements have similar physicochemical properties, interactions may occur between them at separate stages of their respective pathways but, at customary levels of exposure, each system has an accumulative specificity and selectivity which discriminates effectively for the delivery of each metal to its respective depots and functional sites. Copper as a free ion is only found in systems when there is an excessive body burden; the resultant toxicity is a situation that, in healthy animals, is not caused by high dietary intakes of copper.

Cuprous copper is unstable and poorly soluble at systemic physiological pH; it is easily oxidised to the cupric state but these properties are controlled and exploited biologically when the element is bound in complex such as a cuproprotein. In these complexes, copper is usually bound to nitrogen and sulfur ligands provided by the amino acids histidine and cysteine. Cupric copper favours a planar configuration with 4N ligands, whereas cuprous ions form tetrahedral complexes involving 4S ligands (Greenwood and Earnshaw, 1998).

More complex structures involving two or more copper atoms and others with Cu(III) and Cu(IV) states are known but, as yet, their functions have not been fully characterised. An exception to this generalisation is haemocyanin which is found in molluscs and arthropods, including crustaceans. Haemocyanin contains two Cu(I) atoms that interact to bind molecular oxygen reversibly at the low environmental oxygen tensions and temperatures at which these creatures live; it is not found in mammals in which haemoglobin has the same role.

Genomic analyses indicate the existence of many potential copper binding motifs for which the proteins and functions have yet to be identified. Many cuproproteins found in mammals, birds and fish are highly conserved in earlier life forms, thereby occasioning speculation that, similar to iron, copper may have been functionally important before an aerobic environment evolved.

The effect of copper on the composition and the metabolic activity of gut microbiota is reported in detail in Section 3.5 ('Copper intake and effects in gut microbiota profile').

3.1.1. Copper intestinal uptake and transfer

The entry of copper into the food chain may be limited by the redox potential, alkalinity, and sulfur and molybdenum content of the soil. Thus in some areas, copper deficiency may be endemic in grazing livestock despite copper being abundant in the soil. The intraluminal solubility of ingested copper and thus its availability for intestinal uptake is enhanced by an acid milieu; its availability for intestinal absorption can be limited by intraluminal and intraruminal complexation with molybdenum and sulfur mediated by microbiota (see Section 3.4.2) and also by interactions with magnesium, calcium, inorganic and organic phosphate (e.g. phytates) and other dietary components including amino acids, and Maillard reaction products (Section 3.4.2). Furthermore, intestinal uptake may be limited by competition at mucosal uptake and transfer sites with other transition metals. A high exposure to zinc may block the transfer of copper from the gut by inducing enterocytic metallothionein (MT) which sequesters copper in the intestinal epithelium. Vitamin C, by oxidising cuprous copper to cupric, also impairs copper uptake in animal models, but this does not necessarily happen in general. Thus, although a major determinant of the intestinal uptake and transfer of copper, and its systemic (predominantly hepatic) retention is the animal's need for copper, it is also evident, particularly for ruminants, that the efficiency of the absorption of ingested copper can be affected very much by feed composition (Section 3.4.2).

Copper is released from the dietary matrix by gastric acidity and proteolytic activity. Gastric mucosal uptake and transfer of copper do occur but this is probably not a major route in normal physiology. The predominant site for uptake of copper in mature animals is the duodenum and proximal jejunum; however, in young animals, the uptake of copper extends throughout the small intestine, and it is postulated that, in some species, the large bowel might be able to take up copper.

The first stage of copper uptake is its aggregation by the mucus and glycocalyx on the enterocytic microvilli, whence it reaches membrane-associated reductases (six-transmembrane epithelial antigen of the prostate 2 (STEAP2) and the duodenal cytochrome B (Dcytb)) which convert any residual cupric to cuprous copper for translocation into the enterocyte. There are at least two mechanisms for the energy-independent influx of copper into cells such as enterocytes: (i) one involving at least two membrane-bound copper transporters (CTR), one of which CTR1, is a high-affinity carrier, and CTR2 which is a low-affinity transporter; this mechanism is thought to account for 70% of copper uptake and (ii) a second mechanism which accounts for the rest of copper uptake at the gut mucosa is the divalent metal transporter (DMT1). The CTR-dependent mechanism involves the uptake and delivery of cuprous copper into the enterocyte and it is thought possible that the process may involve endocytosis. However, this has not been confirmed, and neither have the other elements of copper trafficking in enterocytes, hepatocytes and other cell types.

There are several proteins involved in copper ADME which are present in most cell types. CTRs, for example, appear ubiquitously to mediate the uptake of copper. They are homologous to a varying extent with proteins with similar functions in bacteria and other species. Within cells, there is an equally ubiquitous depot protein: the MT. The MT comprises a number of monomeric isoproteins each of which has a molecular mass of 6,500 Da and comprises 60 amino acids, about 30% of which are cysteine. These enable a molecule of MT to bind 6–10 atoms of copper. The MT is induced among other things by endotoxemia, infections, calorie restriction, glucocorticoids, exercise, oestrogens and hypothermia, as well as by zinc and probably by high exposure to copper. Thus MT may have roles as a sequestrator of excess copper and as a mobile depot of the metal which adapts to need and stressors. Two other intracellular enzymes, adenosine triphosphate (ATP) ATP7A ATPase (ATP7A) and ATP7B ATPase (ATP7B), are important in transfering copper to apoenzymes, or into vesicles for export from the cell. Although both are ubiquitous, ATP7A predominates in the kidneys, lungs, blood–brain barrier, gastrointestinal tract and muscle. ATP7B predominates in the hepatocytes where it is responsible for the synthesis of ceruloplasmin, and for the excretion of copper into the bile, and there is little ATP7A in the liver. Within cells, both ATP7A and ATP7B are usually distributed around the nucleus where they donate copper to apoenzymes in the trans Golgi network (TGN). Although there are a number of intracellular target-specific chaperone proteins that take copper to its functional sites, their roles are not fully understood.

In the enterocyte, copper is either bound to MT or to a chaperone which translocates the copper to ATP7A for vesicular transfer of the metal across the basolateral membrane into the portal circulation.

Copper is transported in the portal circulation to the liver and then to the systemic circulation. In the portal circulation, copper has a number of carriers; these include a copper transport protein, transcuprein and non-specific carriers such as albumin and amino acids (usually histidine, threonine and glutamine). Usually, nearly all absorbed copper is taken up into the liver within 2 h of ingestion via hepatocytic cell membrane-bound metallo-reductases and CTR1. Copper complexed with phosphatidic acid and fatty acids (FA) has been found in mesenteric lymph; this implies that some copper may bypass the liver and reach the systemic circulation via the thoracic duct.

The liver is the primary depot for copper and the principal mediator of its systemic homeostasis. In the hepatocyte, copper joins a high turnover labile pool, probably centred on copper–glutathione (GSH) complexes from which the metal is distributed to key sites such as:

1) a MT pool and
2) chaperones for delivering copper to the synthesis of cuproenzymes such as zinc–superoxide dismutase (Zn-SOD), mitochondrial inner membrane cytochrome *c*-oxidase and to ATP7B near the TGN for incorporation into apoproteins of cupric enzymes and to form ceruloplasmin, which is then secreted into the circulation or excreted directly in the bile.

The loss of function of ATP7A and ATP7B is responsible for specific disturbances of copper kinetics; for example, in humans, the absence of functioning ATP7A is associated with Menkes' disease and that of ATP7B with Wilson's disease (De Bie et al., 2007). Phenotypically similar diseases are reported in

rodent models and in the case of Wilson disease,[8] other species also, particularly in certain breeds of sheep and dogs (Haywood et al., 2004; Wu et al., 2016).

3.1.2. Copper distribution

Copper is probably distributed to peripheral tissues by complexes similar to those which are formed in the portal circulation. In plasma, 60–70% of copper is found in ceruloplasmin, 10–30% is associated with a transport protein transcuprein, and 15–20% is bound to albumin and amino acids. However, the reported distributions vary between species and it is not clear what the biological significance of this observation might be. Ceruloplasmin had long been thought to be a systemic transport protein for copper but this is probably not so; inherited deficiency of ceruloplasmin (acaeruloplasminaemia) has little effect on copper trafficking and function, but has an appreciable effect on iron utilisation. Albumin has a terminal binding site for copper which lends some specificity to this function. In dogs, however, this site is absent but with little effect on systemic copper utilisation. Dogs (and mice) also have less circulating ceruloplasmin, and it has been suggested that in dogs, the systemic distribution of copper depends on transcuprein and low molecular complexes.

Peripheral tissues are thought to take up and utilise copper in the same way as the liver. ATP7A is essential for the adequate distribution of copper across the blood–brain barrier. In tissues, CRT2 has been localised with lysosomal vesicles and it is surmised that CTR2 is involved in the recycling of copper from hydrolysed protein transporters of copper.

3.1.3. Copper excretion and homeostasis

Systemic homeostasis of copper is effected by hepatobiliary excretion, sequestration in MT pools (predominantly in the liver) and by reduced intestinal uptake and transfer. At customary exposures the retention of absorbed copper in the body is regulated by hepatobiliary excretion which accounts for 98% of copper excretion, and the rest is lost via the urine. Hepatobiliary excretion involves ATP7B, and in a minor capacity ATP7A, secreting copper as a variety of complexes into vesicles. These merge with the hepatocytic apical plasma membrane and transfer the copper complexes into the bile for elimination. The excreted copper appears not to be reabsorbed and is lost in the faeces. The faecal pool of excreted copper also contains copper from shed enterocytes and degraded ceruloplasmin. At times of increased need for copper (e.g. with increased tissue synthesis or low intakes) hepatobiliary excretion of copper is reduced, and intestinal uptake and transfer are increased. Studies at the cellular level show that when there is an increased need for copper, both ATP7A and ATP7B become associated with the TGN and CTR1 increases in several tissues and aggregates near the plasma membrane; in the enterocytes, CTR1 aggregates at the apical membrane.

With excessive dietary copper exposure, the three components of homeostasis become progressively more involved. As hepatobiliary excretion increases, hepatic depots sequestering copper in MT increase and the enterocytic carriers mediating the uptake of copper from the gut are downregulated. High copper states in hepatocytes induce a migration of ATP7B from the TGN to cytosolic vesicular compartments that support biliary elimination of copper. In enterocytes and other cells increased copper leads to the endocytosis and degradation of CTR1, as well as the re-localisation of ATP7A and ATP7B to cytosolic vesicles at the basolateral plasma membrane for transfer of copper into the blood. At estimated intakes of copper above 100 mg/kg body weight (bw), homeostasis is also mediated by the induction of MT in the gut mucosa which blocks the transfer of copper into the portal circulation (Bremner, 1998, Stern et al., 2007). These adaptive phenomena have been deduced from a variety of studies in different models, and the exposure–response relationship has not been fully characterised. There are some inter- and intraspecies differences: for example, the hepatic excretion of copper is relatively less in cattle and sheep than in other species, and ruminants have higher liver copper contents per unit weight than humans or pigs. These aspects are considered further in subsequent chapters/sections of this scientific opinion.

3.2. Copper in animal nutrition

Major differences in copper metabolism exist between non-ruminant and ruminant animals (NRC, 2005) which lead to differences on copper requirements and, particularly, maximum tolerance levels. Although not completely understood, interspecies differences on copper metabolism seem to be related

[8] For more information on Wilson disease, see http://www.omim.org/entry/277900 and http://www.omim.org/entry/309400

to great differences in the ability to excrete copper in bile and the subcellular hepatic copper distribution as discussed in Section 3.1 (Bremner, 1998; Dameron and Harrison, 1998; Haywood et al., 2001).

Non-ruminant species (particularly pig and poultry), which have an adequate copper biliary excretion and most of the copper is bound to MTs in the liver, can tolerate high levels of dietary copper, with hepatic copper accumulation starting at high copper intakes (up 50-fold the requirements). In these species, copper can be used at very high concentrations, as e.g. growth promoters.

On the contrary, ruminants have a very limited capacity for copper biliary excretion and only a very small proportion of copper is bound to MT in the liver. This is particularly true in sheep, where dietary copper in excess of requirements does not appear to increase biliary copper excretion (Saylor and Leach, 1980; Bremner, 1998), leading to very high copper deposition in the liver. Once this storage capacity is overloaded, a sudden and generally fatal haemolytic crisis occurs.

The metabolic behaviour of copper in fish and crustaceans is less well understood. However, it can be assumed that in fish copper follows the same principle as described for monogastric species, except absorption where also that of waterbond copper occurs via the gills. In crustaceans, the respiratory pigment, haemocyanin, is based on copper, which underlines the importance of copper for crustaceans.

In addition, copper interactions with other dietary constituents are essential to determine copper requirements. In ruminants, the ratio of Cu:Mo in the diet is even more important to establish copper requirements than the dietary copper concentration itself (Dias et al., 2014). The range of dietary copper concentrations required by ruminants (particularly sheep) may overlap under certain conditions the dietary concentrations that cause toxicosis. In pig and poultry, species in which copper can be used at high doses as growth promoter (see Section 3.2.4), the competitive interaction with zinc (also used at high concentrations as growth promoter) and the extensive use of other additives that improve zinc availability, mainly phytases, deserves special attention for dietary formulations.

The determination of copper concentrations in liver is the best biomarker for diagnosing copper disorders in animals (Cano-Sancho et al., 2014) as there is no blood parameter/end point able to detect animals at risk of suffering from both copper deficiency and chronic subclinical copper accumulation (López-Alonso et al., 2006), although serum or plasma copper concentrations are used at the farm level for that purpose. Serum/plasma copper concentration represents both the transport pool (containing copper in albumin- and amino acid-bound forms, transported from the intestine to the liver) but mainly (60–70%) the functional pool (corresponding to copper exported from the liver to tissues as ceruloplasmin). However, there are several reasons why serum/plasma copper concentrations are not reliable biomarkers of copper status: (i) ceruloplasmin concentrations are highly influenced by factors other than dietary copper, particularly by inflammation as it is an acute phase protein; (ii) ceruloplasmin concentrations do not increase once the animal has reached an adequate copper status – thereafter, copper accumulates in the liver without showing any detectable change at the blood level, and it is only when the copper storage is overloaded when a sudden release of copper occurs in the blood; and (iii) in situations where important dietary sulfur and molybdenum interactions occur (particularity in ruminants, see also Section 3.4.2), insoluble copper thiomolybdates in the blood do not reflect a real copper deficiency situation. Alternatively, serum ceruloplasmin has been demonstrated to be helpful in situations where excess molybdenum causes insoluble copper thiomolybdate accumulation in the plasma; however, some ceruloplasmin is lost during clotting, making serum values approximately 10–20% lower than those in the corresponding plasma. All the above-mentioned limitations could result in findings that (i) some animals may have serum/plasma copper or ceruloplasmin concentrations within the adequate range but at low hepatic copper concentrations (Herdt and Hoff, 2011), or (ii) animals with low serum copper concentration, considered to be deficient, or in the lower margin of the reference interval, have been found to be at or near a toxic state upon necropsy or hepatic biopsy (Blakley and Hamilton, 1985; López-Alonso et al., 2006). Alternative to liver, copper concentrations in hair/fleece have been proposed as long-term biomarkers for copper, particularly at suboptimal copper intake (Suttle, 2010); however, their usefulness to detect excessive copper accumulation is limited (Blakley and Hamilton, 1985; Arnhold et al., 1998; López-Alonso et al., 2006; Herdt and Hoff, 2011).

3.2.1. Requirements, allowances and recommendations for dietary copper in target animals

The following definitions are used in the context of this section:

Requirement: the individual demand for copper under defined conditions.

Allowance/Recommendation: estimate of the copper supply necessary to meet the average gross demand of the population under common conditions plus a safety factor considering the individual variability, varying bioavailability and interactions between nutrients.

Requirements and allowances are provided by scientific bodies; recommendations by the industry or private bodies. Regarding copper nutrition, the differentiation between requirement and allowances is difficult to distinguish between scientific bodies.

3.2.1.1. Poultry

Copper requirements/allowances and recommendations for poultry categories are presented in Table 2. The National Research Council (NRC) provides the most comprehensive list of detailed requirement values for various classes of poultry (NRC, 1994). Even though NRC values are based on research studies conducted more than 30 years ago and poultry industry/production (and consequently nutritional requirements) has largely changed, NRC copper requirements are still considered adequate (Applegate and Angel, 2014), being the most used reference for researchers. Background copper concentrations in standard commercial diets usually cover the NRC (1994) requirements (Guo et al., 2001; Leeson and Summers, 2005; Luo et al., 2005; Lu et al., 2010; also see Annex C, Table C.1). In the last years, research into copper supplementation has been almost exclusively focused on the growth-promoting effect of copper at concentrations well above requirements (see Section 3.2.4).

NRC copper requirements for chicken reared for laying were 4–5 mg Cu/kg diet; no values were given for laying hens. Copper requirements for meat birds were 8 mg Cu/kg diet even though copper was missing in tabular data for broiler breeders. Copper requirements for turkey were 6–8 mg Cu/kg diet but no specific requirements were provided for geese or duck. Within the European Union (EU) national scientific bodies, copper requirements/allowances for all poultry are in the same dimension as those provided by the NRC (5 and 10 mg Cu/kg diet); the values provided by the Instytut Fizjologii I Zywienia Zwierzat (IFZZ) for turkeys are at least double of the other values.[9] EU Feed Industry recommends 25 mg Cu/kg diet (maximum allowed concentration in animal feed in the EU legislation) for all poultry species and categories. The FEEDAP Panel checked also the nutrition recommendations of some breeder companies (Ross, Lohman and ISA) for copper supplementation; they are largely in line with the recommendations provided by the industry.

Table 2: Copper requirements/allowances and recommendations for poultry (mg/kg complete diet)

Species	Category	Age, production stage	Requirements and allowances				Recommendations	
							EU Feed Industry[(d)]	
			NRC[(a)]	GfE[(b)]	MTT	IFZZ[(c)]	Total	Supplemented
Chicken	Layer	0–6 weeks	5	7	10	5–6	25	12–16
		6 weeks-first egg	4	6	5–10	6–8	25	12–15
		Laying hen	?[(e)]	7	5–10	5–8	25	10–15
		Breeder hen	?				25	
	Fattening	0–8 weeks	8			7	25	13–17
		Starter/Grower/ Finisher	8	7	8–10	7	25	13–17
Turkey	Fattening	0–4 weeks old	8	15	10	20	25	13–16
		4–8 weeks old	8	6	10	20	25	13–16
		8–12 weeks old	6	6	5	20	25	13–16
		12–24 weeks old	6	6	5	25	25	13–16
	Breeder	Laying	8		8	20	25	13–16
		Not laying	6		8	20	25	13–16
Geese						5–8	25	12–15
Duck	Fattening						25	12–15

[9] According to information provided to EFSA, the higher figures provided by IFZZ are based on body weight gain.

Sources: NRC, USA (1994); GfE, Germany (1999, 2004); MTT Finland (2013); IFZZ, Poland (2005); European Feed Industry.
(a): Requirements. Corn–soybean meal-based diets without phytase activity. Layers: 11.912.1 MJ metabolisable energy (ME)/kg; broilers: 13.4 MJ ME/kg; turkey: 11.7–13.8 MJ ME/kg.
(b): Requirements. mg/kg dry matter (DM).
(c): For 'Chicken', Supplementation rate: lower value, minimum addition; higher value, safety margin included. For 'Turkey', Allowance levels.
(d): Data provided by stakeholders following a call for data.
(e): As reported.

3.2.1.2. Pigs

Copper requirements/allowances and recommendations for pigs are presented in Table 3. As in poultry, copper requirements have not been studied in detail in modern pig breeds, up-to-date feeding conditions and diet composition. Thus, most recommendations are based on the same few published results (NRC, 2012). The necessary dietary copper level may depend on diet composition and may be influenced, due to antagonistic interactions, by the levels of zinc, iron, phytate and other nutrients (Carlson et al., 2007; Kumar et al., 2010) (see Sections 3.4.1 and 3.4.2).

The NRC (2012) provides tables on copper requirements using two principles: dietary requirement (expressed as mg Cu/kg feed) or daily requirement (mg Cu/day assuming a specified daily feed intake). The values presented in the current report are given as dietary requirement (copper concentration in feed) but it may be reasonable to focus also on the daily copper requirement, especially in weaned piglets, in which the feed intake is often very low during the first few days after weaning and it may be necessary to take this into consideration as recommended by the NRC.

The minimum copper requirements are 6 mg/kg in young piglets and about 3–4 mg/kg feed in growing-finishing pigs and are defined as the minimum copper requirement (Suttle, 2010; NRC, 2012). Copper requirements are not well documented in pregnant and lactating sows but most studies show that the requirement is estimated to be 10–20 mg/kg feed in reproductive sows, highest during lactation (NRC, 2012). NRC (2012) recommends 5 mg Cu/kg diet to boars.

Within the EU national scientific bodies, the basic copper allowance is generally about 6 mg/kg for piglets and growing-finishing pigs; some countries specify the same dietary level for reproducing pigs although others indicate a slightly higher allowance for reproducing sows (8–10 mg Cu/kg). The Polish allowances (IFZZ) are generally about four times higher for piglets and fattening pigs and about 1.5 times higher for sows compared to the requirements given by NRC, GfE, VSP, MTT and the allowances of Agroscope. In addition to this basic copper requirement, it is common practice to add copper to diets for piglets (170 mg/kg up to 12 weeks of age) because of other functions of copper (see Section 3.2.4). EU Feed Industry recommends 170 mg Cu/kg compound diet for piglets (< 12 weeks) and 25–30 mg Cu/kg compound diet for all other categories (in both cases, the maximum allowed concentration in animal feed in the EU legislation).

Practical diets of piglets in the Netherlands are recommended to contain 12 mg Cu/kg diet (see Bikker et al., 2015). Recently, a Dutch experiment thus aimed to study the effect of 'pharmacological' copper levels above the national copper recommendation by addition of graded copper levels (from 15 to 160 mg Cu (as $CuSO_4$)/kg diet) to diets fed to piglets from day 0 to 56 after weaning at 27 days (Bikker et al., 2015, 2016). As the Bikker studies dealt with dietary copper levels above the recommendation (12 mg/kg diet), the results obtained are addressed in Section 3.2.4 ('Other uses of copper in farm animals').

Table 3: Copper requirements/allowances and recommendations for pigs (mg/kg complete feed)

Category	Body Weight	Requirements and allowances						Recommendations	
		NRC[(a)]	GfE[(b)]	VSP[(c)]	MTT[(d)]	Agroscope	IFZZ[(i)]	EU Feed Industry[(e)]	
								Total	Supplemented
Piglet	< 11 kg bw	6	6	6	6.5–6.8	8	25	170[(f)]	150–160
	11–25 kg bw	5	6	6	6.3–6.7	8	25	170[(f)]	150–160
Pig for fattening	25–50 kg bw	4	4–5	6	6.1–6.3	6	20	25–30	12–17
	50–135 kg bw	3.5/3	4–5	6	5.5–6.1	6	15/12[(j)]	25–30	12–17
Sow		10/20[(g)]	8–10[(h)]	6/6[(g)]	4.9–5.6/ 6.1–6.8[(g)]	9/9[(g)]	15/15	25/25[(g)]	12–16[(h)]

Sources: NRC, USA (2012); GfE, Germany (2008); VSP Denmark (2016); MTT, Finland (2013); Agroscope, Switzerland (2011); IFZZ (2014); European Feed Industry.
(a): Requirements. Corn–soybean meal-based diet with a DM content of 90%. Growing pig: 14.2–13.8 MJ ME/kg; sow and boar: 13.8 MJ ME/kg.
(b): Requirements. mg/kg DM.
(c): Requirements. mg/FU. 1 FU corresponds to 7.7 MJ ME and approximates 1 kg as-fed.
(d): Requirements. mg/MJ net energy.
(e): Data provided by stakeholders following a call for data.
(f): Maximum 170 mg/kg compound feed until 12 weeks of age. Thereafter, max. 25 mg/kg.
(g): First figure or range gestation, second figure lactation.
(h): Combined values for gestation-lactation.
(i): Requirements.
(j): 15 mg/kg feed below 90 kg bw and 12 mg/kg feed above 90 kg bw.

3.2.1.3. Ruminants

Copper requirements/allowances and recommendations for ruminants are presented in Table 4.

Copper requirements for bovine vary between 10 and 22 mg/kg DM (NRC, 2000, 2001). The highest requirements for copper in dairy cows are in close-up period and in the first weeks after parturition. Dry Holstein cows (680 kg bw) 240 days pregnant need 12 mg Cu/kg DM and 279 days pregnant 18 mg Cu/kg DM. Fresh Holstein cows (680 kg bw) with milk production 25 kg need 16 mg Cu/kg DM and with 35 kg up to 22 mg Cu/kg DM (NRC, 2001), which is higher than the average requirement of 11 mg Cu/kg DM for dairy cows (Table 4). Copper requirements of small ruminants differ according to species; higher values are recommended for caprines (15–25 mg/kg DM) and lower for ovines (4–8 mg/kg DM) (NRC, 2007a). Within the EU national scientific bodies, copper requirements/allowances vary in the range of 6–25.2 for bovine, 5–15 for caprine and 5–14.3 for ovine mg Cu/kg diet. EU Feed Industry recommends the maximum allowed concentration in animal feed allowed by the EU legislation for all ruminant species and categories, except for cattle (EU legislation: 35 mg Cu/kg diet) and caprine (25 mg Cu/kg diet).

Table 4: Copper requirements/allowances (mg/kg DM complete feed) and recommendations (in mg/kg complete feed with 12% moisture) for ruminants

Species	Category		Requirements and allowances						Recommendations	
			NRC[(a)]	GfE[(a)]	MTT	Agroscope	CAAS[(a)]	CVB[(b)]	EU Feed Industry[(c)]	
									Total	Supplemented
Bovine	Calves	For rearing	10		10	6	9	14.5	15	10
		For fattening	10		10	6	9	15.2	15	10
	Cows	Dairy cows	11	10	10	10	12	11.1	35	15–35
		Cows for reproduction	12–18	10	10	15	12	25.2	35	15–35
	Cattle		10	8–10	10	10	10	19.1	30–35	20–25
Caprine		Kids for rearing	25	10–15		8	6		10–25	0–15
		Kids for fattening	25	10–15		8	7		10–25	0–15
		Dairy goats	15	10–15		8	5	11.5	10–25	5–15
		Goats for reproduction	15	10–15		8	6	11.6	10–25	5–15
Ovine		Lambs for rearing	5–6			5	6		15	3–15
		Lambs for fattening	5–6			5	7	5.4–10.8[(d)]	15	3–15
		Dairy sheep	4–7			5	8	6.8–13.5[(d)]	15	3–15
		Ewes for reproduction	5–8			5	8	7.2–14.3[(d)]	15	3–15

Sources: NRC, USA (2000, 2001, 2007a); GfE, Germany (1995, 2001, 2003); Agroscope, Switzerland (2006, 2009); MTT, Finland (2013); CAAS, Czech Republic, Zeman et al. (2006); CVB, the Netherlands, Van de Top (2005); European Feed Industry.
(a): Requirements.
(b): Allowances (gross requirements + safety margin 50%).
(c): Data provided by stakeholders following a call for data.
(d): Reflecting breeds of different copper sensitivity; the lowest value derived for highly sensitive breeds.

Unlike poultry and pigs – for which copper requirements have been well established some decades ago and NCR requirements still remain well accepted by the scientific community – in ruminants, particularly in bovine and caprine, an important body of research has been conducted on copper

requirements after the last NRC assessment. Because of its susceptibility to copper deficiency and toxicity, the ovine copper metabolism and requirements had been well studied some decades ago (Corbett et al., 1978; Gooneratne et al., 1979; Saylor and Leach, 1980) and, on this subject, recent research is sparse.

Recent research indicates that cattle could be less tolerant to copper than was traditionally thought (López-Alonso, 2012) and that, in some circumstances, copper requirements could be lower than those proposed by the NRC – high hepatic copper accumulation occurring already below dietary copper concentrations considered safe and recommended by the industry (35 mg Cu/kg diet). Garcia-Vaquero et al. (2011, 2012) compared an unsupplemented control (5–8 mg Cu/kg DM) with a copper-supplemented diet (plus 15 mg Cu from copper sulphate/kg DM) fed for 96 days to cattle for fattening: the authors concluded that routine copper supplementation is not necessary to maintain an adequate copper status in feedlot cattle or to improve zootechnical parameters; histochemistry showed signs of pathological changes in the liver at copper supplementation. In the same way, as little as 20 mg Cu/kg DM (background 4.9 mg Cu/kg DM) could reduce feed intake and feed/gain ratio in finishing steers, as shown by Engle and Spears (2000a). Mullis et al. (2003) recommended the addition of 7 mg Cu/kg DM to diets of Angus and Simmental heifers on the basis of experiments with supplementation of 0, 7 or 14 mg Cu/kg DM. The control diets containing 4.4 (Angus) or 6.4 (Simmental) mg Cu/kg DM did not meet Cu requirement of either breed during gestation and lactation or growth. On the contrary, the supplementation of 30–40 mg Cu/kg DM (background 4.7–5.8 mg Cu/kg DM) in some studies improved performance of bulls, feed/gain ratio and growth, and decreased the prevalence of lameness (Fagari-Nobijari et al., 2013; Netto et al., 2014). Numerical discrepancies among studies could be related to the different concentrations of copper antagonists in the diet. The most important are sulfur and molybdenum which reduced copper absorption due to formation of thiomolybdates in the rumen. More details on Cu-antagonists are given in Section 3.4.2.

In the opposite way, recent research indicates that caprine could have higher copper requirements than traditionally thought and, in some circumstances, could get benefits from copper supplementation above the NRC requirements. Positive effects of copper supplementation for 1-year-old cashmere goats (supplement: 10 mg Cu/kg DM; background: 7.4) on growth and nutrient digestibility were demonstrated (Zhang et al., 2008). For 2.5-year-old wethers, supplementation of a feed (background: 5.6 mg Cu/kg feed) with 20 mg Cu/kg DM enhanced growth, but 30 mg/kg had an inverse effect (Zhang et al., 2009). Low level of copper supplementation (7 mg Cu/kg DM; background: 12.8) had no beneficial effect on goats (Sanjivani et al., 2014). Higher copper levels (10–40 mg Cu/kg DM, supplemented to diets with 6–10 mg Cu/kg) increased body weight, average daily gain (ADG) in goat kids (Datta et al., 2007; Mondal et al., 2007a). Solaiman et al. (2006a,b) and Cummins et al. (2008) observed that oral administration of 100 mg Cu/day for 98 days to goat kids (4–5 months old, fed a basal diet with 13.8 mg Cu/kg) increased ADG and feed:gain ratio, but 200 mg Cu/day decreased ADG. A wide spectrum of copper levels (20, 40, 80, 160, 320, 640 mg Cu from copper sulphate/kg DM; background: 14.3) was evaluated by Huang et al. (2013, 2014) in Jianyang Big-ear goat kids. Copper supplementation did not affect ADG, average daily feed intake (ADFI) and feed:gain ratio of goat kids.

In general, the more recent data reviewed above are in accordance with the established data on ruminant requirements. However, studies in caprines could be interpreted as indicating a somewhat higher requirement, which would likely result from interactions between copper and Cu-antagonists.

3.2.1.4. Horses

The most recent copper requirements/allowances and recommendations for horses are presented in Table 5. Both NRC (2007b) and the Institut National de la Recherche Agronomique (INRA) (2012) had recently reviewed copper requirements of horses, recommending a general value of 10 mg/kg DM feed for all ages of horses, regardless of the degree of work and stage of production. On the contrary, other EU national scientific bodies established different copper requirements for horses depending on breed, age, sex and pregnancy status within the range of 7–12.5 mg/kg DM feed. EU Feed Industry recommends for horses as total dietary concentration the maximum currently authorised copper in animal feed (25 mg Cu/kg diet).

No recent papers on this subject which would allow an update on horse copper requirements have been published.

Table 5: Copper requirements and allowances (mg/kg DM complete feed) and recommendations (mg/kg complete diet) for horses

Category	Age, production	Requirements and allowances				Recommendation EU Feed Industry[(d)]	
		NRC[(a)]	GfE[(b)]	INRA	MTT[(c)]	Total	Supplemented
Growing	4–24 months	10	10–12	10		25	10–15
Stallion		10	7–10	10		25	10–15
Mare	Pregnant, lactation	10	8–10	10	12.5	25	10–15
Adult	No work–heavy work	10	7–10	10	6–12.5	25	10–15

Sources: NRC, USA (2007b); GfE, Germany (2014); INRA, France (2012); MTT, Finland (2013); European Feed Industry.
(a): Requirements. Adult horse with 600 kg bw.
(b): Requirements.
(c): Given as daily requirement, re-calculated for 500 kg bw using feed intake data.
(d): Data provided by stakeholders following a call for data.

3.2.1.5. Rabbits

The NRC (1977) established copper requirements for growing and fattening rabbits at 3 and 5 mg Cu/kg feed for lactating does, whereas INRA (1989) set copper requirements at 5 mg Cu/kg for both categories. Data on recommendations from single publications range from 5 to 30 mg Cu/kg feed, with higher values suggested for breeding does and fur production (Schlolaut, 1987; Halls, 2010; Mateos et al., 2010). EU Feed Industry recommends for rabbits as total copper dietary concentration the maximum currently authorised for this species (25 mg/kg diet).

3.2.1.6. Fish

Fish absorbs copper via two routes: the gills and the intestine; hence, copper is available from both sources: water and the diet. However, copper in the rearing water alone cannot meet the requirements and oral administration of copper is essential for aquatic animals (Lee and Shiau, 2002; Lin et al., 2008; Grosell, 2012). The relative contribution of waterborne copper to whole-body copper content typically accounts for about 10% and increases up to 60% at a dietary copper concentration of 0.8 mg/kg (Kamunde et al., 2002).

A considerable body of research has been carried out allowing to establish the dietary copper requirements for several fish species, such as rainbow trout and common carp, channel catfish, tilapia, grouper, yellow catfish, grass carp, blunt snout bream, large yellow croaker, tongue sole (Wang et al., 2015), red sea bream and Russian sturgeon. Details of these studies (including latin names and end points are presented in Table 6). Overall, dietary requirements for fish range from 3 to 14 mg Cu/kg DM feed, depending on the species, life stage and feeding regime (Lorentzen et al., 1998; Clearwater et al., 2002; NRC, 2011; Mohseni et al., 2014). NRC (2011) has established the copper requirement for Atlantic salmon at 5 mg/kg DM. A detailed description of studies on fish requirements is given in Appendix A.

Table 6: Copper requirements of different fish species

Common name Latin name	Requirement (mg/kg DM)	End point	Reference
Rainbow trout *Oncorhynchus mykiss*	3	Growth rate	Ogino and Yang (1980)
Common carp *Cyprinus carpio*	3	Growth rate	
Channel catfish *Ictalurus punctatus*	5	Cytochrome *c* oxidase and Cu-Zn-SOD activities	Gatlin and Wilson (1986)
Atlantic salmon *Salmo salar*	5		NRC (2011)
Tilapia *Oreochromis* spp.	5	Growth rate, body copper	Shiau and Ning (2003)

Common name Latin name	Requirement (mg/kg DM)	End point	Reference
Grouper *Epinephelus malabaricus*	4–6 ($CuSO_4$)	Growth rate, body copper, Cu-Zn-SOD activity, TBARS[(a)]	Lin et al. (2008)
	2.4–3.7 (organic copper peptide)		Lin et al. (2010)
Yellow catfish *Pelteobagrus fulvidraco*	3.1–4.2	Body copper	Tan et al. (2011)
Grass carp *Ctenopharyngodon idella*	4.7–5.0[(b)]	Growth rate, feed efficiency, ceruloplasmin activity	Tang et al. (2013)
Blunt snout bream *Megalobrama amblycephala*	12–14	SGR[(c)]	Shao et al. (2012)
Large yellow croaker *Larimichthys croceus*	3.7–7.6	Growth rate, body copper, vertebrae copper, Cu-Zn-SOD activity	Cao et al. (2014)
Tongue sole *Cynoglossus semilaevis*	11–12	Growth rate, protease-, amylase-, lipase-, Cu-Zn-SOD and lysozyme activity	Wang et al. (2015)
Red sea bream *Pagrus major*	4.5 (nanoparticles)[(d)]	Feed efficiency ratio, protein efficiency ratio, protein retention, body copper, protease activity, lysozyme activity and total serum protein	El Basuini et al. (2016)
Russian sturgeon *Acipenser gueldenstaedtii*	6.8–8.2	Growth rate, body copper, Cu-Zn-SOD and ceruloplasmin activity	Wang et al. (2016)

(a): TBARS: thiobarbituric acid-reactive substances (hepatic levels).
(b): In mg/kg wet weight.
(c): SGR: specific growth rate.
(d): Supplemented as copper nanoparticles (Sigma-Aldrich, 207780-500G: 99%, USA).

3.2.1.7. Crustaceans

Few data are available on the copper requirements of crustaceans; the most studied farmed crustaceans to date are various species of shrimp.

The respiratory pigment in crustaceans is haemocyanin, which has an analogous role to haemoglobin. It has been estimated that 40% (wet weight) of the body copper content in shrimp is found in haemocyanin (Depledge, 1989). Consequently, crustaceans have a higher physiological demand for copper than vertebrates.

Davis et al. (1993) fed juvenile *Penaeus vannamei* semi-purified diets containing 0 (unsupplemented), 4, 8, 16, 32, 64 or 128 mg Cu/kg diet (as copper sulphate) for 42 days. The copper content of the feeds was not analytically confirmed; however, the unsupplemented feed contained 2 mg/kg diet. The water content of the basal diet was not measured but was formulated to achieve a moisture content of 8–10%. The copper requirement was reported to be 34 mg/kg diet based on growth.

Wang et al. (1997) reported a dietary copper requirement of 25 mg Cu/kg diet in fleshy prawn (*Penaeus chinesis*) based on growth and tissue mineralisation. Liu et al. (1990) established a copper requirement of 53 mg/kg in *Penaeus orientalis* based on growth, tissue mineralisation and cytochrome *c* oxidase activity.

An 8-week feeding trial was conducted to determine the dietary copper requirement and its effect on the non-specific immune responses of juvenile grass shrimp (*Penaeus monodon*) (Lee and Shiau, 2002). Purified diets with seven levels of copper (1.0, 13.3, 25.1, 36.4, 47.2, 92.7 and 178.2 mg Cu/kg DM from copper chloride) were tested. Shrimp fed diets containing 13.3 and 25.1 mg Cu/kg DM had significantly higher weight gain, feed efficiency and protein efficiency ratio than shrimp fed the unsupplemented control diet and diets containing 47.2, 92.7 or 178.2 mg Cu/kg DM. Total haemocyte count was higher in shrimp fed diets with 13.3–36.4 mg Cu/kg DM diet than shrimp fed diets with higher copper levels, or the unsupplemented diet. Intracellular superoxide anion production ratios were significantly higher in shrimp fed diets containing 13.3–36.4 mg Cu/kg than shrimp fed the 178.2 mg Cu/kg DM. The adequate dietary copper concentration in growing *P. monodon* was 17–23 mg Cu/kg DM based on growth and about 13–36 mg Cu/kg diet DM for non-specific immune responses.

Sun et al. (2013) exposed juvenile Chinese mitten crabs (*Eriocheir sinensis*) to graded dietary copper levels (1.9, 12, 21, 40, 80 or 381 mg/kg diet, as copper sulphate) for 8 weeks. Crabs fed with 21 and 40 mg Cu/kg diet had significantly greater weight gain than crabs fed all other diets. Haemolymph oxyhaemocyanin contents were highest in crabs fed the 21 and 40 mg Cu/kg diet, followed by crabs fed the 12 and 80 mg Cu/kg diet, and lowest in crabs fed the 1.9 and 381 mg Cu/kg diet. Activity of Cu-Zn SOD, phenoloxidase and total haemocyte count was significantly higher in crabs fed the 21 and 40 mg Cu/kg diet than in crabs fed the 1.9 and 381 mg Cu/kg. This study indicates that the optimum level of dietary copper for Chinese mitten crab is between 20 and 40 mg/kg wet weight.

Bharadwaj et al. (2014) studied the effects of graded levels of dietary copper from an inorganic (copper sulphate) vs an organic source (copper chelated to a hydroxy analogue of methionine) in Pacific white shrimp (*Litopenaeus vannamei*) in a 6-week feeding trial. Shrimp generally required three to four times more dietary copper from copper sulphate than from a chelated copper source to attain comparable growth. Growth rates of shrimp fed 168 and 286 mg Cu/kg from copper sulphate were significantly higher than the control group (9 mg Cu/kg). In comparison, the shrimp fed the chelated copper had significantly higher growth rates at exposure concentrations of 59, 75 and 96 mg Cu/kg compared to the control group.

Kong et al. (2014) examined the effects of copper supplementation (as copper sulphate) on growth and antioxidant activities in juvenile oriental river prawns (*Macrobrachium nipponense*). Prawns were fed semi-purified diets containing 2.8, 12.2, 20.9, 29.8, 43.1, 78.9 or 157.1 mg Cu/kg for 8 weeks. Weight gain was significantly higher and feed conversion ratios (FCR) significantly lower in prawns in the 2.8–78.9 mg Cu/kg dietary groups compared to the group fed the 157.1 mg Cu/kg diet. Copper concentrations in the hepatopancreas, muscle and whole body increased with increasing dietary copper level. Hepatopancreas Cu-Zn-SOD activity, glutathione peroxidase (GSH-Px) and total antioxidant competence were highest in prawns fed the 43.1 mg Cu/kg diet. The dietary copper requirement in juvenile oriental river prawns was approximately 27 mg/kg based on whole-body copper retention.

The effects of copper supplementation on growth, digestive enzyme activities and biochemical constituents in freshwater prawn post larvae (*M. rosenbergii*) were investigated by Muralisankar et al. (2015). Prawns were fed diets containing 0.8 (unsupplemented), 11.8, 22.7, 40.6, 66.5 or 88.3 mg Cu/kg DM for 90 days. Survival, growth and digestive enzyme activities (protease, amylase and lipase) were significantly higher in prawns fed the 11.8, 22.7 or 40.6 mg Cu/kg diet DM compared to the other groups.

Muralisankar et al. (2016) studied the effects of diets supplemented with copper nanoparticles (as copper chloride) on growth, digestive, antioxidant and metabolic enzyme levels, and non-specific immune response of freshwater prawn post larvae (*Macrobrachium rosenbergii*). Prawns were fed diets containing 11.6, 22.5, 33.3, 54.9, 76.5 or 98.1 mg Cu/kg DM for 90 days. Survival, SGR, digestive enzyme activities (protease, amylase and lipase), and total and differential haemocyte counts were significantly higher in prawns fed the 22.5 mg Cu/kg diet DM compared to the other groups.

Considering the inconsistent data set with large interspecies variability, the FEEDAP Panel is not in a position to conclude on the copper requirements of crustaceans, but it is thought to be considerably higher than in other target animal species.

3.2.1.8. Dogs and cats

The copper requirements/allowances and recommendations for dogs and cats are presented in Table 7. As published experiments on copper requirements are very scarce, NRC (2006) only published requirements for kittens and included allowances for all other categories of dogs and cats. No published scientific papers deal with this subject since the latest copper requirement/allowance update for dogs and cats (NRC, 2006).

Table 7: Copper requirements/allowances (mg/kg DM feed) for dogs and cats

Species	Category	Requirements and allowances			
		NRC[(a)]	NRC[(b)]	GfE[(a)]	FEDIAF[(c)]
Dog	Puppies after weaning		11.0	11.3	11.0
	Late gestation, lactation		12.4	10.6	11.0
	Adult, maintenance		6.0	6.6	7.2–8.3[(d)]

Species	Category	Requirements and allowances			
		NRC[(a)]	NRC[(b)]	GfE[(a)]	FEDIAF[(c)]
Cat	Kittens after weaning	4.5	8.4		10.0
	Late gestation, lactation		8.8		10.0
	Adult, maintenance		5.0		5.0–6.7[(e)]

Sources: NRC, USA (2006); GfE, Germany (1989); FEDIAF (2014).
(a): Requirements.
(b): Allowances.
(c): Data provided by stakeholders following a call for data. http://www.fediaf.org/fileadmin/user_upload/PetNutrition/FEDIAF_Nutrition_Guideline161214.pdf
(d): Depending on maintenance energy requirements; the lower level (95 kcal/kg$^{0.75}$) indicating the higher copper requirement.
(e): Depending on maintenance energy requirements; the lower level (75 kcal/kg$^{0.67}$) indicating the higher copper requirement.

3.2.2. Copper deficiency symptoms

The relative susceptibility of different copper-dependent processes, and therefore the manifestations of copper deficiency, varies with the animal species and the stage of development at which critical dysfunctions develop (Suttle, 2010). Within the domestic animals, ruminants are particularly susceptible to copper deficiency and copper-related disorders (both primary and secondary); for the other domestic species, copper concentrations in the diets generally cover nutritional requirements. A wide range of copper-related diseases has been associated with low copper status (Suttle, 2010). The summary given below follows in large parts the review of Suttle (2010), in which specific references can be found.

Following some pioneering field studies in the 1930s, swayback was eventually reproduced experimentally by feeding pregnant ewes diets low in copper or high in molybdenum and sulfur. Three types of ataxia, the most sensitive clinical consequences of copper deficiency, occur naturally in lambs: (i) neonatal (paralysis or ataxia at birth, followed by death; primary anoxic lesions in the brain stem and demyelination of the cerebral cortex); (ii) delayed (uncoordinated hindlimb movements, stiff and staggering gait, swaying hind quarters, often triggered by flock disturbances); and (iii) atypical (older lambs stand transfixed, head quivering and apparently blind; primary lesion, cerebral oedema). In the goat kid, the delayed form is predominant and cerebella hypoplasia is an additional feature. Calves undergo a slow regular myelination of the central nervous system (CNS) and do not succumb to neonatal or delayed ataxia. Copper-deficient chickens may also display ataxia and spastic paralysis (Leeson, 2009). Some clinical cases of copper deficiency were described in the recent literature. Ozkul et al. (2012) described congenital copper deficiency in goats – swayback disease. The kids with a twisted carpal joint were identified; the animals walked on their articulus carpii and newborn goats had stiff legs. The determined mean plasma copper levels of goats that gave birth to kids with congenitally twisted carpal joints were generally decreased (9.6 ± 0.4 μmol/L; 2.99–11.8 μmol/L). Sousa et al. (2012) described an outbreak of enzootic ataxia among sheep. The symptoms began 30 days after birth, with a clinical condition that included locomotion difficulty, limb ataxia, tremors and continual falls. Copper concentration in the plasma (5.9 ± 1.1 μmol/L) and liver (4.1 ± 2.5 mg/kg DM) was low but without any difference between clinically healthy animals and those affected by enzootic ataxia. Despite the low copper content of the diet, the high hepatic iron levels (1,375 ± 325 mg/kg on DM) suggest that the antagonic effect of iron may have been an important factor in triggering copper deficiency in these animals.

When copper deprivation occurs in sheep after myelination is complete, it is the fleece that displays abnormality: wool crimp is progressively lost until fibres emerge almost straight, giving rise to 'stringy' and 'steely' wool with diminished tensile strength and elastic properties. The zonal changes are irreversible, but copper supplementation quickly restores normal properties in new wool growth. Changes in the growth and physical appearance of the hair have also been reported in cattle. In breeds of cattle with highly pigmented coats, loss of coat colour (achromotrichia) is usually the earliest and sometimes the only clinical sign of copper deprivation. White wool develops in normally black-woolled sheep and pigmentation is so sensitive to changes in copper intake that unpigmented bands can be produced by intermittent copper deprivation. Pigmentation of feathers in colour-feathered strains is reduced (Hill and Matrone, 1961). A lack of hair pigmentation has also been described in horses.

Anaemia due to copper deficiency was described first in poultry (Elvehjem and Hart, 1929). Iron absorbed as Fe(II) is usually transported as Fe(III), a conversion which requires ferroxidase enzyme, a

component of which is copper. Anaemia develops after severe or prolonged copper deprivation. In lambs it is hypochromic and microcytic in type, similar to the anaemia of iron deficiency, whereas in cows and ewes it may be hypochromic and macrocytic. Hansen et al. (2010) found that severe, long-term copper deficiency in growing beef calves reduced iron status due to reduced export of iron from the liver. Copper deficiency affected hepatic gene expression of hepcidin and ferroportin, but did not affect duodenal expression of proteins important in iron metabolism. Nutritional anaemia induced by copper deficiency has been demonstrated in piglets fed diets very low in copper but is easily alleviated by increased dietary supply of copper (NRC, 2012). Anaemia has also been described in horses.

Abnormalities in bone development vary widely both within and between species. The disturbances of endochondral ossification that give rise to uneven bone growth (osteochondrosis) can only affect growing animals and bone morphology will be influenced by the rate of growth, body weight distribution, movement and even by the rate of hoof growth at the time of copper deprivation. Widening of the epiphyses of the lower-limb bones is a common manifestation in growing cattle. Young chicks became lame within 2–4 weeks when fed a copper-deficient diet. Bones are fragile and easily broken, the epiphyseal cartilage becomes thickened and vascular penetration of the thickened cartilage is markedly reduced (Leeson, 2009). Bone disorders (osteochondrosis, lowered osteoclast activity, bowing of legs) are also reported to be due to copper deficiency in young pigs probably related to defects in cartilage formation of bones and joints as seen in ruminants. Osteochondrosis and osteodysgenesis have been largely associated with hypocupraemia in horses (Carbery, 1978; Bridges et al., 1984). In foals, copper deficiency causes severe degenerative disease of the cartilage (copper is a required co-factor of lysyl oxidase, an enzyme needed for collagen synthesis) characterised by breaking of articular and growth plate cartilage through the zone of hypertrophic cells, and resulting in arthritis and periarticular enlargement of the long bones (Eamens et al., 1984; Bridges and Harris, 1988; Bridges and Moffitt, 1990). When foals were fed a liquid milk-replacer diet containing 1.7 mg Cu/kg DM for 13–16 weeks, lameness was observed 2–6 weeks after serum copper concentrations had decreased to less than 0.1 μg/mL (Bridges and Harris, 1988). Feeding foals with 8 mg Cu/kg diet, as compared to 25 mg Cu/kg diet, resulted in declining liver copper values, osteochondritis, epiphysitis and limb deformities over a 6-month period (Hurtig et al., 1993). Secondary copper deficiency associated with excessive chronic intake of zinc is devastating in rapidly growing foals, as in this species zinc is a potent inhibitor of copper gut absorption (Cymbaluk and Smart, 1993).

Natural clinical manifestations of connective tissue dysfunction are rare. Osteochondrosis in young farmed deer has been attributed to copper deprivation, is accompanied by gross defects in the articular cartilages and may arise through impaired collagen and elastin development. Subperiosteal haemorrhages and imperfect tendon attachments are seen in lambs on molybdenum-rich pastures (Hogan et al., 1972; Pitt et al., 1980). Copper deficiency in birds, and especially in turkeys, can lead to rupture of the aorta (Graham, 1977). The biochemical lesion in the copper-deficient aorta is likely related to failure to synthesise desmosine, the cross-link precursor of elastin; the lysine content of copper-deficient elastin is three times that seen in control birds, suggesting failure to incorporate lysine into the desmosine molecule. An apparent relationship between low blood copper concentrations and uterine artery rupture in aged parturient mares suggests reduced copper absorption with age or reduced ability to mobilise copper stores (Stowe, 1968).

The copper-responsive diarrhoea widely reported in grazing cattle has been induced experimentally in hypocupraemic Friesian calves. 'Teart scours' occur before liver or blood copper reaches subnormal levels and they may be caused by acute, localised, thiomolybdate-induced copper depletion of the intestinal mucosa. In contrast to young ruminants, copper deficiency is not reported to induce scouring in pigs (NRC, 2012).

Poor growth is a common feature of copper deprivation in grazing sheep, cattle and deer. Growth retardation in the field is usually associated with mild exposure to molybdenum and, similar to infertility, is mostly a feature of molybdenum-induced copper deprivation under experimental conditions. Furthermore, growth can be retarded in the absence of any clinical abnormalities and is accompanied by poor feed conversion efficiency when molybdenum-induced copper deprivation begins *in utero*. Hansen et al. (2009) observed lower body weight gain (BWG) till weaning in beef calves with copper deficiency (plasma copper 1.5–6.4 μmol/L) in comparison with adequately supplemented (plasma copper 13–24 μmol/L); however, the BWG of the two groups did not differ during the growing phase and in the finishing phase. Enjalbert et al. (2006) conducted a retrospective study using analysis of plasma copper from 2,080 dairy and beef cow herds and concluded that inadequate copper status (plasma copper below 8 μmol/L) did not increase the risk of low fertility or other reproduction disorders, but increased the risk of health disorders or growth retardation in calves. In conclusion,

copper deficiency is not observed in ruminants when the diet contains 6–8 mg Cu/kg DM with the exception of increased concentration of antagonists (sulfur, molybdenum, iron) in the diet.

The lack of copper supply may result in poor growth and performance and the pig has been suggested as a model to study copper deficiency in humans as artificially designed diets low in copper resulted in lowered metabolism of simple sugars in pigs (Scholfield et al., 1990).

Clinical signs of copper deficiency in other animal species share the main symptoms described above, but not all, probably due to lesser investigations.

Copper deficiency in rabbits will manifest as retarded growth, grey hair, bone abnormalities and anaemia, among other symptoms (Mateos et al., 2010). However, even at the lower levels of supplemental dietary copper, no deficiency symptoms are expected because of the high copper content of most raw materials used in rabbit feeds (e.g. sunflower meal, alfalfa, wheat germ feed, grape marc/pulp; Annex B, Tables B.1, B.2).

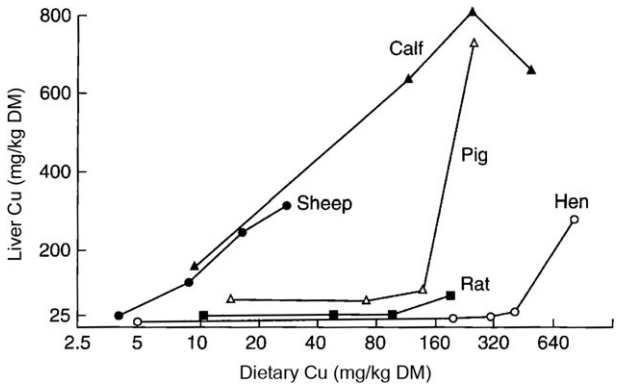
Fish fed diets with copper contents below requirement and reared in water with low copper concentrations may develop deficiency symptoms. Decreased tissue copper concentrations, suppressed appetite, reduced growth, anaemia and reduced activity of copper-containing enzymes have been observed in copper-deficient fish (Ogino and Yang, 1980; Gatlin and Wilson, 1986; Shiau and Ning, 2003; Lin et al., 2008; Tan et al., 2011; Chen et al., 2015b). Copper deficiency signs including mortality, cataracts and caudal fin erosion were observed in rainbow trout after 12 weeks of feeding on a plant-based diet without copper supplementation (Read et al., 2014). Dietary copper deficiency (in terms of poor growth, enlargement of the heart and reduced copper levels in the hepatopancreas and carapace) was observed in Pacific white shrimp (*L. vannamei*) fed semi-purified diets with < 34 mg/kg feed (Davis et al., 1993).

Copper deficiencies are rare in dogs and cats. Zentek and Meyer (1991) fed a low dietary level of copper (1.2 mg Cu/kg DM) to beagle puppies from 1 to 6 months of age. Plasma copper concentrations began to fall after 4 weeks on the diet. After 3 and 4 months of depletion, a loss of hair pigmentation on the face and head and hyperextension of the distal phalanges occurred, respectively. Studies of copper deficiency in cats showed clinical signs different from those observed in the dog. Doong et al. (1983) found that kittens fed less than 4 mg Cu/kg DM showed signs of copper deficiency that included decreased growth rate and reduced concentrations of copper in the liver but no other consistent clinical signs were observed. Adult female cats fed low dietary copper levels (3 or 6 mg Cu (as cupric sulphate)/kg DM added to a basal-purified diet containing 0.8 mg Cu/kg diet) showed reduction of reproductive efficiency, increasing the time for successful conception compared to control cats fed diets supplemented at 10 mg Cu/kg diet (Fascetti et al., 1998, 2000).

3.2.3. Tolerance of animals to dietary copper

As previously stated, large differences exist among animal species in their tolerance to dietary copper. These differences are mainly related to the capacity for copper excretion in the bile as well as the way in which copper is bound within the hepatic cell. Figure 1 exemplifies the interspecies differences in hepatic copper accumulation at different concentrations of dietary copper. Ruminants (particularly sheep, although recent information also includes cattle; López-Alonso et al., 2005a,b) have a very limited capacity for biliary copper excretion and at dietary copper concentrations slightly above the requirements (twofold), copper starts to accumulate at high concentrations in the liver. On the contrary, monogastric species (including pig, poultry, horses and possibly rabbits) can largely increase biliary excretion and copper does not start to accumulate in the liver until copper concentrations approximately two orders of magnitude over the nutritional requirements are given.

Once inside the hepatocyte, large interspecies differences exist on the mode copper is bound. Ruminants have a very limited capacity for MT synthesis and only a small proportion of copper can be safely stored. Once MT are saturated, copper starts to accumulate in the nucleus and as free ions in the cytosol, leading to oxidative damage. On the contrary, in monogastric species most of the copper (up to 80%) is safely stored in the liver cell bound to MT (Bremner, 1991).



The figure accurately shows end points for liver copper in separate experiments in which dietary copper was varied: those end points reflect how long each study ran. With cumulative toxicants such as copper, liver copper goes on increasing unless and until adaptive mechanisms limit the rise: they generally do in cattle but not sheep (Suttle, 2016).

Figure 1: Species differences on responses in liver copper deposition to increases in dietary copper supply as summarised by Suttle (1987). Taken from Suttle (2010)

3.2.3.1. Poultry

Although it is well documented that levels at 20 times the requirements can provide benefits to health and/or performance of poultry (see also Section 3.2.4), toxicity signs do not occur until the dietary levels approach 100 times the requirement. Unlike ruminants, no major feed ingredients used in poultry nutrition are sufficiently high in copper to cause toxicity. Naturally occurring cases of copper toxicity are quite rare, most often due to accidental overdoses of copper sulphate or other copper sources (Malinak et al., 2014).

Based on published literature, NRC (2005) set the copper maximum tolerable level (MTL) at 250 mg Cu/kg diet for chickens and turkeys and at 100 mg Cu/kg diet for ducks. Using graded levels of supplemental basic copper chloride (150, 250, 500, 750 and 1,000 mg Cu/kg diet; basal diet — with 5 mg Cu/kg diet as copper sulphate — contained 15 mg Cu/kg diet), Persia et al. (2004) suggested toxic levels of copper (estimated by regression analysis) to be 642 mg Cu/kg in terms of weight gain and 781 mg Cu/kg for feed utilisation. The same authors also suggest a breed effect in response to toxic levels of copper, because New Hampshire × Columbian chicks are more tolerant to high levels of dietary copper compared with commercial (Ross × Ross) broiler strains.

To the knowledge of the FEEDAP Panel, no more recent studies have been conducted to evaluate copper tolerance in poultry. However, in countries where copper is allowed at high doses in animal feed, many studies have been conducted over the years aiming to evaluate the effect of copper as growth promoter and to reduce cholesterol levels in eggs and meat products (see Section 3.2.4).

None of the experiments in chickens for fattening found negative effects on feed intake and body weight when copper was added up to 450 mg/kg diet (Guo et al., 2001; Luo et al., 2005; Mondal et al., 2007b). However, when other end points are considered, negative effects can be observed. Some studies with broilers have indicated that supplementation at 250 mg Cu/kg diet can cause slight gizzard lining erosion (Robbins and Baker, 1980), proventriculitis (Wideman et al., 1996), lesions in the oral cavity, tongue and pharynx (Chiou et al., 1999) or reduced blood haemoglobin and packed cell volumes (Samanta et al., 2011a). In long-term studies with laying hens, 400 mg Cu/kg diet, or higher, reduced feed intake and egg production (Güçlü et al., 2008), and increased gizzard weight (Jackson et al., 1979; Jackson and Stevenson, 1981). Supplementation with 500 mg Cu/kg diet reduced body weight and feed intake, and caused gizzard erosion in turkey poults (Christmas and Harms, 1979) although in older turkeys, addition of up to 500 mg Cu/kg diet did not affect performance (Leeson et al., 1997). Ducklings accumulated more copper in their livers than chicks when fed high dietary copper concentrations (Wood and Worden, 1973) and thus, may be more sensitive to copper toxicosis. Addition of 500 mg Cu/kg to duckling diets caused myopathy characteristic of selenium-vitamin E deficiency (Van Vleet, 1982). High mortality and severe necrosis in the gizzard, skeletal muscle and intestine were observed in ducklings fed 1,000 mg Cu/kg diet.

In conclusion, the FEEDAP Panel considers that the current NRC (2005) MTLs for chickens and turkeys (250 mg Cu/kg diet) and ducks (100 mg Cu/kg diet) remain still valid.

3.2.3.2. Pigs

Pigs are among the most tolerant animal species to dietary copper. This is because they have a great capacity for copper biliary excretion and it is not until copper is provided in the diet at very high concentrations that pigs start to accumulate copper in an appreciable way (Figure 2). Once the liver capacity to deliver copper is overloaded, toxicity appears, with reduced haemoglobin levels and jaundice being the most common signs of copper toxicity in this animal species (NRC, 2005, 2012).

It is well assumed that supplementation of 250 mg Cu/kg diet increases liver copper concentration without causing negative effects on animal growth or health. On the contrary, the addition of such high copper concentrations in the diet (as copper sulphate) results in increased growth and thus, copper has been extensively used as growth promoter in the pig industry in the last six decades (NRC, 2012). Consequently, hepatic copper accumulation has been extensively studied in pigs receiving increasing graded copper doses. Figure 2 summarises the results of deposition of copper in pig liver from 12 studies conducted at various copper concentrations in the diet, which include eight publications considered by SCAN (2003). A polynomial regression curve was fit (see Figure 2); the summary output of the model is provided in Appendix B. At low dietary copper levels (more than onefold the physiological requirements), copper concentrations in the liver do not increase appreciably; it is at 100–150 mg Cu/kg diet when copper starts to accumulate in the liver.

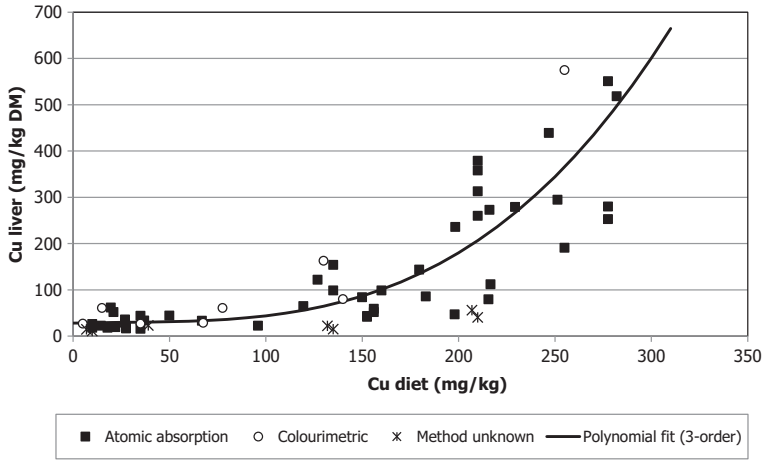


Figure 2: Copper deposition in liver of pigs. Data from the following publications were used: Lucas and Calder (1957), Allen et al. (1961), Elliot and Amer (1973), Omole and Bowland (1974a,b), Castell et al. (1975), Hansen and Bresson (1975), Cromwell et al. (1978), Braude (1980), Madsen et al. (1982), Bradley et al. (1983), Hernandez et al. (2008). Various symbols denote different analytical methods used in the studies. A polynomial regression curve of order 3 was fit ($y = 28.0048 + 0.1032x - 0.0011x^2 + 0.00005x^3$; $R^2 = 0.6868$)

According to the regression curve, the liver of pigs when fed with 25 and 170 mg Cu/kg feed would contain 29.6 and 116.9 mg Cu/kg DM, respectively; these values refer to pigs which are until a slaughter weight of between 77 and 107 kg (initial bw 7–25 kg) with the copper concentrations reported in Figure 2.

Four studies reported liver copper concentrations of piglets (Apgar et al., 1995; Schiavon et al., 2000; Zhao et al., 2014; Bikker et al., 2015) after feeding different copper levels for 5–8 weeks. As an average of five experimental groups with 150–282 mg dietary Cu/kg (the data of Zhao et al. (2014) were not considered because of inconsistencies), a copper concentration of 48 mg/kg liver DM was calculated. A comparison of this value with the regression-derived value shows the influence of feeding time on liver copper. The most recent study provides information on the liver copper content when piglets are fed dietary copper up to 166 mg/kg and until slaughter with about 120 kg body weight with 10–15 mg Cu/kg feed (Bikker et al., 2015). The control group (20 mg Cu/kg in the piglet phase) showed 39.7 mg Cu/kg liver DM, the high copper group (166 mg Cu/kg in the piglet phase) showed 49.6 mg Cu/kg liver DM. It is concluded that feeding piglets up to 170 mg Cu/kg feed followed by a reasonably reduced copper concentration (e.g. 10–15 mg/kg) would not result in essentially increased liver copper levels compared to continuous low copper feeding.

NRC (2005) has established an MTL of copper for pigs at 250 mg/kg based on several studies in growing pigs fed diets supplemented with up to 500 mg Cu/kg diet. Pigs receiving 500 mg Cu/kg diet had reduced growth and haemoglobin concentrations (Bunch et al., 1965; Kline et al., 1971) and may also result in increased mortality (DeGoey et al., 1971).

The duration of exposure to copper has a major impact on toxic reactions (NRC, 2012). A study with sows over four reproductive cycles showed an increase in the culling rate of sows fed 116 mg Cu (from copper sulphate pentahydrate)/kg compared to the control group (unsupplemented diet, 8 mg Cu/kg), mainly due to lack of pregnancy after mating (Poulsen, 1993). These results indicate that long-time dietary copper supplementation of about 100 mg/kg — which is normally considered to be a safe concentration for pigs — may negatively affect the reproductive capacity in sows (Poulsen, 1993). Similar reproductive failure has been observed in gilts (Poulsen, 1993). In contrast, a long-lasting study with sows (up to six reproductive cycles) found no effect on the reproductive performance when fed diets supplemented with 250 mg Cu (from cupric oxide)/kg (Cromwell et al., 1993). The differences may be due to the different copper sources used in the two studies as cupric oxide is known to have a lower bioavailability (see Section 3.4.1).

Copper toxicity may also be accentuated or alleviated by the dietary composition, applied feedstuffs, and elements such as phytate and other minerals affecting the availability and absorption of copper (see also Section 3.4.2.). Thus, increased zinc and iron levels are reported to prevent copper toxicity (Suttle, 2010; NRC, 2012). Moreover, it is speculated that pigs restricted in feed intake may not be as sensitive to high copper doses as *ad libitum* fed pigs, indicating that the daily intake may also affect copper tolerance in pigs (NRC, 2012).

In conclusion, pigs are tolerant to high dietary copper supply; a copper supply of up to 250 mg/kg diet is generally considered to be the maximum tolerable concentration. However, the duration of exposure, the dietary contents of, for example, iron and zinc, the copper source and other factors may affect the exact upper limit.

3.2.3.3. Ruminants

Ruminants, particularly sheep, are very sensitive to dietary copper. Both clinical and subclinical episodes of chronic copper poisoning (CCP) represent nowadays the main toxicological problem in this animal species, being responsible for important economic losses over the world (see review of López-Alonso, 2012).

The CCP in ruminants is a two-stage process (Howell and Gooneratne, 1987). The first stage is the prehaemolytic phase, during which copper accumulates in the liver without clinical signs of disease being manifested. Only when the liver's capacity to accumulate copper is overloaded, usually following a stressful event of any nature, does substantial liver degeneration develop. Copper is then released from the liver, usually causing a haemolytic crisis (Howell and Gooneratne, 1987). The clinically silent phase of the disease makes diagnosis difficult until the animal experiences a haemolytic crisis. In fact, in a study of herds with clinical copper toxicosis, the clinical cases represented only a small proportion of the actual cases of copper poisoning (Laven et al., 2004). Identification of animals in the silent, chronic phase of copper accumulation is important not only to prevent economic losses due to subsequent severe disease or death, but also to avoid the subclinical disease, and to adapt copper supplementation to physiologic needs (López-Alonso et al., 2006).

The narrow margin between the copper physiological needs and the copper concentrations at which toxic effects can occur makes the establishment of MTLs in ruminants a difficult task. Overall, the great negative interactions between copper and sulfur and molybdenum, as well as iron (which form insoluble copper or iron thiomolybdates in the rumen, and consequently determine copper absorption) and the limited biliary excretion makes that ranges for copper requirements and copper toxicity can overlap under certain conditions.

The mechanisms of the three-way interaction between copper, sulfur and molybdenum were first described by Suttle (1975) and are described in detail in Section 3.4.2. Suttle (2010) stated marginal bands for CCP for sheep 12–36 mg Cu/kg DM, for cattle 50–300 mg Cu/kg DM and for goats 30–100 mg Cu/kg DM (values below the lower limit rule out CCP; values above the upper limit are strongly suggestive of CCP).

Although the above-mentioned difficulties were well identified in its last review of 2005, the NRC has estimated MTLs of copper at 100 mg/kg DM for beef cattle, 40 mg Cu/kg DM for dairy cattle and 15 mg/kg DM for sheep (NRC, 2005).

Reports of copper poisoning were somewhat rare; however, more recently, an increasing number of episodes of copper toxicity have been reported in cattle (Bidewell et al., 2000; VLA, 2001). Bidewell

et al. (2012) investigated the occurrence of CCP in a British dairy herd and concluded that dietary copper of 40–50 mg/kg DM can cause CCP. Cattle given copper feed concentrations within the EU-authorised levels can accumulate excessive copper concentrations in the liver; at hepatic copper concentrations slightly higher than normal (around 125 mg/kg wet weight), negative effects on animal performance in terms of reduced feed intake and ADG (Engle and Spears, 2000a) and liver damage (Garcia-Vaquero et al., 2012) were observed. These studies indicate that cattle can, in certain circumstances, be less tolerant to copper than originally thought. The increased risk of CCP in cattle was not necessarily associated with an increase in dietary copper, but could be related to changes in the bioavailability of copper supplements (i.e. from cupric oxide to other more available sources) and diets with a high proportion of concentrate feed (feedlots) where copper is more available (López-Alonso, 2012; more details are provided in Section 3.4.1).

Cattle are at greatest risk of developing CCP prior to weaning when given copper-rich milk substitutes (Suttle, 2010); this is because young ruminants have higher copper absorption and are more susceptible to copper toxicosis than adults. Croubels et al. (2001) described chronic copper intoxication in veal calves after a mixing error resulted in copper levels ranging from 120 to 159 mg Cu/kg in the milk powder. The calves were fed about 8 L of milk per day over a period of 3 weeks. The first clinical sign was haemoglobinuria and four calves died. Six months after the use of the milk replacer was terminated, other calves that had been exposed to copper still showed distinct growth disturbances. Although less studied than in sheep, genetic differences among breeds in copper metabolism also occur in cattle. Simmental accumulates less copper in the liver than Angus (Ward et al., 1995; Mullis et al., 2003), with biliary copper excretion being higher in the former (Gooneratne et al., 1994). Jersey cows are more susceptible to copper toxicity than Holstein cows (Du et al., 1996) but the latter accumulate more copper in the liver than Galician blonde (Miranda et al., 2010). These results indicate that the sensitivity to excessive copper supply is affected by breed; however, these differences between cattle breeds are considered minor and of no practical relevance.

Sheep is the most susceptible species to copper. Complete sheep diets containing more than 15 mg Cu/kg DM can cause CCP. Sheep show histological or biochemical evidence of liver damage at liver copper levels of 350 mg/kg DM. The first phase of CCP (prehaemolytic) is characterised by copper accumulation in the liver (up to 1,000–1,500 mg Cu/kg DM) and increased liver enzymes (glutamate dehydrogenase, GLDH; γ-glutamyltransferase, GMT); the second phase is marked by an acute haemolytic crisis (Suttle, 2010). Although the molecular mechanisms behind the sheep sensitivity to copper toxicosis are not completely known, it is assumed to be due to their inability to increase biliary copper excretion in response to high copper intake. In addition, large differences exist between breeds to accumulate copper in liver when fed low (Littledike and Young, 1993; Suttle et al., 2002) or moderate to high dietary copper (Woolliams et al., 1982) and, thus, ovine breeds have been classified as tolerant or resistant to copper deficiency/toxicity. Van den Top (2005) summarised breed differences in the hepatic copper accumulation of sheep; he concluded that Texel breed has a high tendency to accumulate copper and, on the other extreme, the Scottish Blackface breed always has the lowest hepatic copper and therefore may be the most susceptible to copper deficiency.

The CCP in sheep results from an excessive supplementation of copper (particularly when animals received large amounts of concentrates; Suttle, 2010) or the use of feeds that have been contaminated with copper from agricultural or industrial sources. Copper toxicity has been extensively reported in both sheep and cattle that have been grazing on pastures grossly contaminated with copper-enriched pig slurry (Parkinson and Yells, 1985; Christie and Beattie, 1989; Poole et al., 1990; López-Alonso et al., 2000).

Finally, recent studies confirm that goats appear to be more tolerant to copper than originally thought. Kids (4–5 months old) tolerated copper doses of 100 mg/day for 98 days; 200 mg/day (but not 100 mg/day) resulted in a significant increase in malondialdehyde in mesenteric adipose tissue (Cummins et al., 2008). Solaiman et al. (2006a,b) supplemented copper (0, 100 and 200 mg Cu/day) from copper sulphate to goat kids for 14 weeks (basal diet 13.8 mg Cu/kg DM; ADFI: 1–1.2 kg). The copper concentration in liver was 206, 504 and 778 mg/kg DM, respectively. Although both copper doses did not cause any signs of toxicity, copper concentration in the liver of both supplemented groups was in the limits (407–1,017 mg Cu/kg DM) stated by Suttle (2010) as being risky for the development of CCP. Huang et al. (2013) did not find any signs of copper toxicity in Jianyang Big-ear goats at the highest tested dose (640 mg Cu/kg DM given for 96 days); however, copper concentration in the liver increased from 502 mg Cu/kg DM (supplementation 40 mg Cu/kg DM) to 839 mg/kg DM (supplementation 640 mg Cu/kg DM).

In conclusion, the current NRC (2005) MTLs for cattle and goats of 40 mg Cu/kg DM and for sheep of 15 mg Cu/kg DM are valid assuming normal dietary concentrations of molybdenum (1–2 mg/kg DM) and sulfur (1.5–2.5 g/kg DM) (see Section 3.4.2). The FEEDAP Panel notes that these copper MTLs may cause CCP when the feed contains lower levels of copper antagonists (molybdenum, sulfur) than anticipated above.

3.2.3.4. Horses

Little information exists in the literature concerning copper tolerance in horses. It seems that copper toxicity in horses is extremely rare. Horses are relatively tolerant to high dietary copper concentrations. NRC (2005) has established an MTL of copper for horses at 250 mg/kg feed, based on a study conducted in pony mares fed 791 mg Cu/kg diet for 183 days. Despite final liver copper concentrations of more than 3,000 mg/kg DM, no adverse clinical signs were observed in the mares or their foals (Smith et al., 1975). Although the report of Smith et al. (1975) provides evidence that MTL would be substantially higher, the scarce research data, as well as the possible genetic difference in susceptibility to copper toxicity, make it reasonable the MTL established by NRC.

3.2.3.5. Rabbits

The NRC (2005) established the MTL for rabbits at 500 mg Cu/kg diet based on two studies (Patton et al., 1982; Grobner et al., 1986) in which animals received 500 mg Cu/kg diet for up to 32 days without adverse effects on growth performance. The addition of 1,000 mg Cu/kg diet reduced weight gain, feed intake and feed efficiency in rabbits (Grobner et al., 1986).

3.2.3.6. Fish

Overloading of copper in fish can be deleterious and triggers growth retardation, histological alterations (Shaw and Handy, 2006) and oxidative stress (Hoyle et al., 2007). Responses to chronic copper exposure via feed or water include increased oxygen consumption, reduced average swimming speed, increased ion regulation, decreased number of lymphocytes and increased neutrophils, effects on the immune system, altered activity of Cu-dependent and -independent enzymes, and proliferation of epithelial cells in gills or intestine (Handy, 2004).

The NRC (2005) stated that MTLs of diet-borne copper for fish is affected by species and fish size or life stage, and set these values at 100 and 500 mg Cu/kg diet for Atlantic salmon and rainbow trout, respectively. Tolerance to dietary copper varies considerably among fish species as described below.

Berntssen et al. (1999a) exposed Atlantic salmon (*Salmo salar* L.) parr to dietary copper sulphate concentrations of 5 (control), 34 or 691 mg Cu/kg complete feed for 4 weeks. Increases in the metal binding protein MT, enterocyte apoptosis and cell proliferation in the intestine occurred in fish exposed to 34 or 691 mg Cu/kg diet. Liver copper accumulation and increased liver MT occurred at the highest dose (691 mg Cu/kg diet). Fish exposed to 34 mg Cu/kg feed had increased intestinal and hepatic lipid peroxidation (evident from TBARS levels), and reduced intestinal α-tocopherol levels. Intestinal and hepatic GSH-Px activity increased in the 34 mg/kg diet copper group; however, tissue selenium and GSH levels decreased. The results from this study indicate that dietary copper exposure affects lipid peroxidation at a relatively low concentration (34 mg Cu/kg diet) in Atlantic salmon (Berntssen et al., 2000). In another experiment, Atlantic salmon fry were fed graded levels of copper – 7 (control), 37, 467, 639, 869 or 1,781 mg Cu/kg dry weight feed – for 12 weeks (Berntssen et al., 1999b); fish fed diets containing 7 and 37 mg Cu/kg had reduced growth and whole-body protein, glycogen and selenium content.

The threshold of diet-borne copper toxicity for Atlantic salmon parr and fry can be set in the range of 15–17 mg Cu/kg bw per day based on organ copper burden and reduced growth (Clearwater et al., 2002). However, based on intestinal lipid peroxidation and increased enterocyte cell turnover (as evident from increased apoptosis and cell proliferation), a threshold level of dietary copper toxicity in Atlantic salmon parr could be as low as 1 mg Cu/kg bw per day (34 mg Cu/kg complete feed).

Dietary toxicity of copper measured as growth inhibition in rainbow trout occurs at approximately 664–730 mg Cu/kg feed corresponding to 44 mg Cu/kg bw per day (Lanno et al., 1985; Julshamn et al., 1988; Clearwater et al., 2002). Campbell et al. (2002) reported increased oxygen consumption, reduced swimming activity and loss of circadian periodicity in rainbow trout following 3-month exposure to 726 mg Cu/kg DM compared to control fish. Haematocrit, red and white blood cell counts, serum protein, glucose and growth were not affected by dietary copper exposure, whereas intestinal and hepatic MT were elevated in copper-exposed fish.

Kim and Kang (2004) investigated the effects of graded levels of dietary copper sulphate (0, 50, 125, 250 and 500 mg Cu/kg; not analytically confirmed) on growth, haematological and biochemical parameters (haematocrit, haemoglobin, total protein, glucose, calcium, magnesium, alanine transaminase (ALT) and aspartate aminotransferase (AST)) in juvenile rockfish, *Sebastes schlegeli* exposed for 60 days. Reduced SGR was observed in fish exposed to copper concentrations above 50 mg/kg. Haematocrit, haemoglobin and red blood cells decreased over the course of the experiment in all dietary groups; however, there were no significant differences among groups. Significant differences in the biochemical parameters were only observed in the highest copper group (increase in total protein, magnesium, AST and ALT).

Yellow catfish (*Pelteobagrus fulvidraco*) were fed 0.83, 4.6 or 102 mg Cu/kg diet DM (as copper sulphate) for 8 weeks (Chen et al., 2015a). Dietary copper level did not significantly affect survival, condition factor, hepatosomatic index, viscerosomatic index or visceral adipose index in the fish. The BWG and SGR were significantly higher in fish fed 4.6 mg Cu/kg DM compared with those fed 0.83 or 102 mg/kg DM diet. Fish exposed to 102 mg Cu/kg DM diet had elevated hepatic copper concentrations and reduced liver lipid content. Histological examination of fish from this treatment group showed pyknotic nuclei in hepatocytes and parenchyma disorganisation. Exposure to elevated dietary copper (102 mg Cu/kg DM diet) reduced the hepatic concentrations of free carnitine, total carnitine and acylcarnitine, as well as the ratio of acylcarnitine/free carnitine, but increased the free carnitine/total carnitine ratio in the liver (Chen et al., 2015b).

The effects of graded levels of dietary copper supplementation on growth, haematological profile and ceruloplasmin activity were studied in Nile tilapia (*Oreochromis niloticus*) fed diets containing 17.2 (unsupplemented), 23.4, 26.9, 29.9, 1,095 or 1,609 Cu/kg DM (as copper sulphate) for 120 days (Damasceno et al., 2016). Growth performance was significantly impaired in fish fed the diets with the two highest copper concentrations, and fish in these groups had significantly reduced red blood cell counts, haemoglobin, haematocrit and total plasma protein levels compared to the control. The highest hepatic copper levels were found in fish fed diets with the high copper concentrations, whereas low level supplementations of the control diet resulting in 23.4–29.9 mg total Cu/kg DM did not significantly affect the copper concentration in the liver. Nile tilapia exposed to 1,968 mg Cu/kg DM for 42 days had reduced feed intake and weight gain by day 21 of exposure, compared to controls (Shaw and Handy, 2006). Dietary copper exposure (as copper sulphate) had no effect on mortality, feed to gain ratio, hepatosomatic index, haematology, gill or intestine pathology; however, increased lipid deposition was observed in the hepatocytes of copper-exposed fish. El-Serafy et al. (2015) reported that Nile tilapia exposed to 2,000 mg Cu/kg diet (not analytically confirmed) for 30 days had significantly increased number of chromosomal aberrations (including centromeric attenuation, break, deletion, fragmentation, gap end to end association, ring, polyploidy and stickiness) in head kidney cells compared with control fish, indicating genotoxicity of copper at high doses in fish.

Hoyle et al. (2007) fed African walking catfish (*Clarias gariepinus*) for 30 days diets with different copper concentrations (16 and 1,495 mg Cu/kg DM, as copper sulphate). High dietary copper reduced significantly feed intake and SGR, but no significant effects were seen in mean body mass, feed to gain ratio or haematology.

Salmonids appear to be less tolerant to excessive levels of dietary copper compared to other fish species. Increased enterocyte apoptosis, cell proliferation, MT induction, as well as increased intestinal and hepatic GSH-Px activity and lipid peroxidation, were observed in Atlantic salmon following dietary exposure to 34 mg Cu/kg diet for 1 month. The current maximum level for copper in feed of 25 mg/kg appears to be protective.

3.2.3.7. Crustaceans

Crustaceans appear to tolerate relatively high levels of dietary copper; however, there is limited information available on a few farmed species of shrimp, and even less on crabs.

Prawn post larvae (*M. rosenbergii*) fed diets containing 66.5 or 88.3 mg Cu/kg DM (as copper chloride nanoparticles) for 90 days had significantly higher SOD, catalase and aspartate transaminase activities in the muscle, and lower growth and survival rates compared to prawns fed 40.6 mg Cu/kg DM (Muralisankar et al., 2016). The authors concluded that 67–88 mg Cu/kg diet DM may be toxic to *M. rosenbergii*.

Feed to gain ratio and growth were significantly lower in oriental river prawns fed a semi-purified diet containing 157.1 mg Cu/kg diet (as copper sulphate) for 8 weeks compared to prawns fed 2.8–78.9 mg Cu/kg diet (Kong et al., 2014). Furthermore, cumulative mortality was significantly higher

in prawns fed 157.1 mg Cu/kg compared to prawns fed 12.2–78.9 mg Cu/kg in a challenge test with *Aeromonas hydrophila* conducted following the feeding experiment.

Diets supplemented with copper sulphate (64, 93, 135, 198, 286 or 430 mg Cu/kg diet) or an unsupplemented diet (9 mg Cu/kg diet) were fed to Pacific white shrimp for 6 weeks (Bharadwaj et al., 2014). Shrimp fed a diet containing 430 mg Cu/kg had significantly lower growth rates than shrimp fed diets containing 198 mg Cu/kg or 286 mg Cu/kg, indicating that copper was toxic at the highest dietary level.

Purified diets with seven levels (1.0, 13.3, 25.1, 36.4, 47.2, 92.7 or 178.2 mg Cu/kg diet DM as copper chloride) of copper were fed to *P. monodon* juveniles for 8 weeks (Lee and Shiau, 2002). Weight gain, feed efficiency, protein efficiency ratio and total haemocyte counts were significantly lower in shrimp fed 47.2, 92.7 or 178.2 mg Cu/kg diet than in shrimp fed diets containing 13.3–36.4 mg Cu/kg diet.

Juvenile Chinese mitten crabs (*E. sinensis*) were fed diets containing 1.9, 12, 21, 40, 80 or 381 mg Cu/kg diet, as copper sulphate) for 8 weeks (Sun et al., 2013). Crabs exposed to either 80 or 381 mg Cu/kg fed had significantly lower weight gain rate (WGR) and haemolymph oxyhaemocyanin content than crabs fed optimal copper levels. Cu-Zn SOD and phenoloxidase activity, and total haemocyte count were significantly reduced in crabs fed diets 381 mg Cu/kg.

In conclusion, studies conducted on several species of shrimp/prawns and Chinese mitten crab indicate that the current authorised maximum level of copper in feed of 50 mg/kg is protective. However, this level may be too low considering the copper requirement in crustaceans which appears to be in the region of 20–40 mg/kg diet (although insufficient data are available to conclude).

3.2.3.8. Dogs and cats

In general, dogs are not particularly susceptible to copper toxicity. However, a specific problem in copper storage can result in some breeds showing symptoms of copper toxicity. Bedlington Terriers (Webb et al., 2002), West Highland White Terriers (Thornburg et al., 1986) and Skye Terriers (Haywood et al., 1988) have been shown to have a hereditary disorder, which causes copper to accumulate in the liver and results in hepatitis and cirrhosis (Hyun and Filippich, 2004). The typical symptoms of toxic levels of copper in liver include lethargy, vomiting, jaundice and weight loss. These breeds, particularly Bedlington Terriers, have been used as models for Wilson disease in humans. Moreover, in the last years, a considerable amount of information on the susceptibility of other breeds to copper hepatopathy has been published, including Doberman Pinschers (Mandigers et al., 2004), Dalmatian (Webb et al., 2002) and, most recently, the Labrador Retriever (Hoffmann et al., 2006; Smedley et al., 2009). Occasionally, other pure dog breeds as well as mixed-breed dogs have been associated with chronic copper toxicity (Watson, 2004). Dogs with this disorder absorb normal amounts of copper, but biliary excretion of copper is reduced as a secondary response to an abnormal hepatic function altering biliary copper excretion (Watson, 2004; Kilpatrick et al., 2014). Progressive accumulation of copper within hepatic lysosomes results in liver damage that can be fatal. The occurrence of a haemolytic crisis is uncommon in dogs with inherited sensitivity to copper toxicosis, but can be seen in terminal stages. Once diagnosed, this type of copper toxicosis is treated by restricting copper intake, adding an antagonist (such as zinc) to the diet to reduce copper absorption, and/or with the use of chelators that increase urinary copper excretion (Hyun and Filippich, 2004).

Apart from the above-mentioned information on copper-susceptible breeds, limited research has examined the level of copper needed to cause toxicosis in normal dogs, which makes it not possible to set an MTL for copper in this animal species.

Unlike the dog, there appears to be little or no information on the toxic effects of copper in cats, making it impossible to set an MTL for copper in this animal species.

3.2.4. Other uses of copper in farm animals

Copper has received considerable attention due to its antimicrobial properties; nowadays it is well known that copper improves performance in pigs and poultry (except laying hens) when fed at pharmacological levels markedly exceeding the minimum requirement (Barber et al., 1955; Smith, 1969; Jenkins et al., 1970; Miles et al., 1998; European Commission, 2003; Jongbloed et al., 2011; Ma et al., 2015). Less demonstrated are the beneficial effects of copper on growth performance in rabbits.

Copper has also been demonstrated to have significant effects on lipid metabolism in many animal species and may thus change the fatty acid profile and reduce the cholesterol content in animal products.

However, due to the increasing public concern regarding bacterial resistance to antibiotics and the possible linked co-resistance to copper (see also Section 3.6), the animal production industry is looking for nutritional components with antimicrobial properties that maintain intestinal health and allow for optimal growth. As such, alternatives to antibiotics and copper have been the focus of many studies with non-ruminant animals over the recent years (Heo et al., 2013).

3.2.4.1. Growth promotion/prevention of diarrhoea

Poultry

In the United States and some Asian countries where copper is allowed at high doses in animal feed, many studies have been conducted in recent years to evaluate copper as growth promoter. The addition of copper levels ranging from 100 to 450 mg/kg (e.g. as copper sulphate, copper citrate, copper chloride) has beneficial effects on body weight and feed intake in chickens for fattening (Guo et al., 2001; Luo et al., 2005; Mondal et al., 2007b; Wang et al., 2007; Ao et al., 2009; Xiang-Qi et al., 2009; Lu et al., 2010; Samanta et al., 2011a,b; Liu et al., 2012; Kim and Kil, 2015).

When comparing the growth-promoting effect of copper, differences have been reported among copper sources. TBCC and cupric sulphate (220 and 180 mg Cu/kg feed, respectively) equally improved carcass weight of chickens for fattening after 45 days of feeding compared to the negative control (30 mg Cu/kg feed) (Arias and Koutsos, 2006). Recently Jegede et al. (2011) reported significant higher daily weight gain with broilers fed copper proteinate than with cupric sulphate supplemented at 50–150 mg Cu/kg feed for 56 days (background 35 mg Cu/kg feed). Jackson and Stevenson (1981) found no effects of feeding 150–750 mg Cu/kg diet (48 weeks) from cupric oxide while adverse effects on body weight and egg production were noted for the same doses of cupric sulphate. More recently, copper supplemented at doses of 65–390 mg/kg feed either from cupric sulphate or TBCC did not affect feed intake, egg production, egg mass, feed to gain ratio and body weight of laying hens during a 16-week trial (Liu et al., 2005). In a more recent study (Pekel and Alp, 2011), no effects on laying performance were found when hen diets were supplemented with 250 mg Cu from cupric sulphate, copper proteinate or copper lysine, compared to the control.

Little information about the growth stimulation mechanisms is available. One of the possible mechanisms has been attributed to the bactericidal and/or bacteriostatic effects of copper on the gastrointestinal tract microbiota (Jensen, 2016; see also Section 3.6.1). The bactericidal action of copper is dependent on the concentration of free ionic copper in solution, while the free ionic copper concentration is affected by pH and solubility (Pang and Applegate, 2007).

Pigs

About six decades ago, the addition of high levels of dietary copper sulphate above the minimum requirement was shown to result in increased growth in pigs. This growth-promoting effect of copper has received much attention (NRC, 2012). However, the mechanisms behind this growth-promoting effect are still not fully understood (Jensen, 1998; Poulsen and Carlson, 2004). One plausible explanation is that the effect is attributed to an antimicrobial effect (less microbial load and less microbial (potential toxic) metabolites in the gut) leaving more nutrients and energy available to the pig itself (e.g. Fuller et al., 1960; Henderickx et al., 1980; Radecki et al., 1992; Jensen, 1998; Corpet, 2000; Jensen, 2016; see also Section 3.6.1). This is further supported by the results of Shurson et al. (1990) who showed that high copper (total 283 mg copper/kg feed) tends to increase daily weight gain and to improve feed:gain ratio and villus height/crypt depth ratio in conventional piglets but showed the opposite effects in germ-free piglets. Other authors claim that the growth-promoting effect of copper in piglets might be a systemic effect rather than an antimicrobial effect in the intestinal tract (Zhou et al., 1994a). Carlson et al. (2007) reported that the addition of 175 ppm copper to piglet diets resulted in increased plasma zinc level irrespective of the dietary zinc level (100 or 2,500 ppm) indicating that copper fed above the requirement improved zinc status due to the interactions between zinc and copper (see Section 3.4.2). Studies conducted over the last two decades suggest that the growth-promoting effect of copper may be ascribed to an up-regulating effect of copper on appetite resulting in an increased feed intake. These results support the idea of a systemic effect as proposed by Zhou et al. (1994a). First, Zhou et al. (1994b) found an increase in pituitary growth hormone messenger ribonucleic acid (mRNA) in piglets fed 214 compared to 15 mg Cu/kg diet. However, Zhou et al. (1994b) emphasise that they could not exclusively relate copper to the increase in growth hormone gene expression as the production of growth hormone is influenced by many other factors. Recent Chinese studies pursued the suggested possible appetite-regulating effect of copper, and

Li et al. (2008) found an increase in neuropeptide Y (NPY) concentration and NPY mRNA expression level in the hypothalamus of pigs fed 125 and 250 compared to 10 mg Cu/kg diet. They concluded that high dietary copper appears to stimulate appetite by enhancing NPY concentration and NPY mRNA expression but, later, Yang et al. (2010) found that, despite an increase in the levels of mRNA expressing growth hormone-releasing hormone of the hypothalamus in pigs fed 125 compared to 5 mg Cu/kg diet, the feed intake was not affected by copper supply of pigs. Zhu et al. (2011) reported that pigs fed diets supplemented with 125 or 250 mg Cu/kg diet (as sulphate) had similar feed intake and performance which was higher than in pigs fed 10 mg Cu/kg diet. At the same time, Zhu et al. (2011) also evaluated the effect of copper supplementation on the expression of some hypothalamic appetite-regulating genes, but observed no significant changes in the mRNA expression due to high dietary copper supplementation; thus, Zhu et al. (2011) concluded that the source of the stimulatory drive increasing feed intake in piglets consuming high copper-supplemented diets needs further investigation.

The growth-promoting effect of copper is generally ascribed to young pigs, whereas no effects are seen in older pigs (European Commission, 2003; NRC, 2012). This is in line with the review of Heo et al. (2013) unfolding the marked changes in gastrointestinal physiology, microbiology and immunology imposed on pigs at weaning. The growth-promoting effect of copper may be affected by the diet composition and dietary concentrations of, for example, zinc and iron that may contribute to the variance in obtained effects of copper as growth promoter (Suttle, 2010; Section 3.4.2).

Hill et al. (2000) compared the effects of 250 mg copper (high copper, as sulphate) and 3,000 mg zinc (high zinc, as oxide) alone or in combination in weanling pig diets in 12 different study locations. They reported that growth and feed:gain increased in piglets fed either high copper or high zinc compared to piglets fed copper and zinc according to requirements, but they found no additive or synergistic effects of feeding the combination of high copper and high zinc to young pigs. Faecal consistency and colour were firmer and darker when piglets were fed either high copper or high zinc but the authors pointed out that the 'reason and relevance of the faecal consistency changes are unknown' as no concurrent effect on the incidence of diarrhoea was observed. The authors concluded that high zinc addition (3,000 mg Zn/kg diet) could be beneficial to pig producers, whereas they recommended discontinuing the tradition of adding high copper doses (as sulphate) to high zinc diets.

Recently, a Dutch trial studied the effect of graded levels of copper by adding 15, 80, 120 and 160 mg copper/kg diet in piglets of more than 12 weeks (Bikker et al., 2015, 2016). The trial aimed to study the effect of dietary copper level above the recommended dietary level (12 mg Cu/kg diet) fed together with 45 mg zinc (as sulphate) on performance and faecal consistency, and found that the highest performance and the lowest percentage of growing pigs with soft faeces was observed when the 160 mg copper/kg diet was supplied for the entire experimental period (until 56 days after weaning). Furthermore, any reduction in level below 160 mg Cu/kg diet or duration of highest copper supplementation resulted in a small but not significant reduction in performance and faecal consistency. Although the faecal consistency was affected by copper level (less soft in pigs fed the high copper level), Bikker et al. (2015) reported no effects of the dietary copper level (addition of 15, 80, 120 and 160 mg copper/kg diet) on the number of required medical treatments. Thus, Bikker et al. (2015, 2016) did not detect a correlation between faecal consistency and number of medical treatments. This is in line with Hill et al. (2000) who reported a firmer consistency and a darker coloured faeces in piglets fed copper above requirements (250 mg Cu as copper sulphate/kg diet) but pointed out that the relevance of the faecal consistency changes is unknown. Furthermore, Bikker et al. (2015) did not detect any clear effect of dietary copper level on mortality, although the difference in mortality between treatment groups was significant ($p = 0.04$). These findings agree with Hill et al. (2000) reporting no difference in diarrhoea in pigs fed 250 mg Cu/kg alone or in combination with 3,000 mg Zn/kg diet in weanling pigs, although they saw differences in faecal consistency and colour.

In conclusion, dietary copper levels above requirements promote growth in piglets but the effects seem not to be related to faecal consistency. No additive effects on pig performance of high copper fed to pigs receiving high dietary zinc are evident but 'growth-promoting' levels of copper may improve the zinc status in pigs.

Rabbits

Several authors have reported that supplementation with 100–400 mg Cu/kg diet improves growth performance in rabbits (Bassuny, 1991; Ayyat et al., 1995; Abo El-Ezz et al., 1996; Onifade and Abu, 1998; Aboul-Ela et al., 2000; Skrivanová et al., 2001). The beneficial effects are more noticeable in

young animals under poor sanitation status and in the presence of digestive diseases such as enteritis and enterotoxaemia (Patton et al., 1982). There are, however, conflicting reports. For example, King (1975) and Harris et al. (1984) did not find any benefit for the rabbit with copper supplementation of 175 and 100/200 mg Cu/kg diet, respectively — copper background in diet not given. Fekete et al. (1988) observed an improvement in the growth of rabbits fed a high-protein diet (180 g crude protein (CP)/kg) when 100 mg Cu/kg was added to the diet (11 mg Cu/kg background), but no effects were observed in the low-protein group (140 g CP/kg, background 9 mg Cu/kg). Aboul-Ela et al. (2000) fed weaned rabbits (4 weeks of age) for 2 weeks two basal diets (high fibre/low energy, low fibre/high energy) with and without 250 mg supplemental Cu (from copper sulphate)/kg feed (background not given). Supplemental copper improved performance significantly only in the high fibre/low energy group.

It is concluded that a potential effect of high dietary copper in rabbits may occur; however, the conditions (feeding/sanitary) favouring the effects are not fully understood.

3.2.4.2. Lipid metabolism

Poultry

Dietary copper has a certain impact on fat saturation of tissues and products in poultry, however less consistent than in other species (for a review, see Leeson, 2009). Moderately high levels of dietary copper (approximately 250 mg Cu/kg diet) have been reported to reduce cholesterol levels in eggs and meat products, although these effects are sometimes but not consistently associated with loss of performance. Copper supplementation at concentrations between 125 and 200 mg Cu/kg diet seems to have a somewhat consistent effect on reducing egg cholesterol content, although the quantitative effect is quite variable (7–25%) depending on the study; it has been postulated that it might be related to the assay procedures and resultant variability in baseline levels of cholesterol found in normal eggs (Ankari et al., 1998; Pesti and Bakalli, 1998; Balevi and Coskun, 2004; Lien et al., 2004; Lim et al., 2006). In a more recent study (Pekel and Alp, 2011), no effects on laying performance and on egg cholesterol were found when hen diets were supplemented with 250 mg Cu from cupric sulphate, copper proteinate or copper lysine, compared to the control.

Comparable reductions as to those above described have been observed in cholesterol and serum lipid profile of tissues and organs of birds. Bakalli and Pesti (1995) recorded a 12% reduction in plasma cholesterol and a 20% reduction in breast muscle of broilers fed a 250 mg Cu/kg diet. Konjufca et al. (1997) showed that feeding a 180 mg Cu/kg diet resulted in a 25% decrease in cholesterol concentration in liver, breast and thigh muscle of broilers and concluded that lower dietary levels of copper cause hypercholesterolaemia by elevating the levels of hepatic GSH-Px. Likewise, Bakalli and Pesti (1995) correlate reduced cholesterol levels with reduced GSH and also reduced CoA reductase. More recently, Samanta et al. (2011b) reported significantly reduced levels of cholesterol and triglycerides in plasma and of cholesterol in meat when broilers were fed (42 days) up to 250 mg Cu/kg feed originating from cupric sulphate, while maintaining improved growth performance compared to the negative control (10 mg Cu/kg feed). Jegede et al. (2011) found that copper proteinate was significantly more effective in promoting growth and reducing plasma cholesterol than cupric sulphate when broilers were fed for 56 days up to 150 mg Cu/kg feed compared to the negative control (35 mg Cu/kg feed).

The cholesterol-reducing effect of high dietary copper in poultry could be an interesting issue for human nutrition. When evaluating this effect, it should also be considered that lower cholesterol would be correlated with higher copper in poultry tissues (particularly liver), and that the currently authorised maximum copper content for poultry feed in the EU (25 mg/kg; see Table 1) would prevent a practical use.

Pigs

A study from the early 1970s also demonstrated an effect of dietary copper on the biosynthetic pathway of deposited triglyceride in pigs but the mode of action of copper on lipid metabolism in pigs remains unclear (Amer and Elliot, 1973). The authors found no effect on the performance of pigs fed 250 mg Cu/kg which increased the proportion of unsaturated fatty acids (UFA) and peroxide value (PV) of the backfat when slaughtered at 23, 46, 69 and 92 kg, compared to the control.

Later studies showed that, despite copper being known to promote oxidation reactions *in vitro*, no detrimental effects on the antioxidative and oxidative status of pig[10] were detected in pigs fed 154 compared to the 15 mg Cu/kg diet from 25 to 100 kg bw (Lauridsen et al., 1999).

Fry et al. (2012) reported an increased duodenal lipid peroxidation and hepatic oxidative stress in weaning piglets fed 250 mg Cu/kg from either cupric sulphate or TBCC, compared to the control (7 mg Cu/kg feed); TBCC tended to cause less oxidative stress in the intestine than cupric sulphate.

In conclusion, the reviewed studies with partially contradictory results do not provide any practical impact with concern to human nutrition. Moreover, the doses used are near to the highest tolerated concentration and its use in pig feeding is not allowed following EU legislation (piglets up to 12 weeks, 170 mg Cu/kg and other pigs 25 mg/kg; see Table 1).

Ruminants

A special area of interest is represented by experiments evaluating the effect of copper supplementation on the lipid metabolism of cattle. Copper supplementation (20–40 mg Cu/kg DM) to high-concentrate finishing diets can decrease subcutaneous adipose tissue deposition and cholesterol concentration, and can increase UFA in beef (see review of Engle, 2011). The mechanism by which copper affects the profile of FA is not clear but it includes effects on biohydrogenation, esterification and mobilisation of triglycerides. Copper has a high potential for reduction and, in the rumen, it can act on reduction reactions, interfering with the microbial biohydrogenation of UFA (Netto et al., 2014). The hypothesis for the decrease of cholesterol assumes that high supplementation of copper indirectly regulates cholesterol biosynthesis by decreasing reduced GSH and increasing its oxidised form, glutathione disulfide (GSSG). The increased concentration of GSSG has shown an indirect relationship with the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) activity (Netto et al., 2014). The above-mentioned changes in lipid metabolism by copper supplementation (20–40 mg/kg DM) are published mainly by one research group (Engle and Spears, 2000b; Engle et al., 2000a,b,c); some recent studies in bulls found a decrease in cholesterol and an increase in the proportion of UFA in meat after supplementation of copper (30–40 mg/kg DM, background 4.7–7) (Correa et al., 2012, 2014; Fagari-Nobijari et al., 2013). On the other hand, these effects were not confirmed in Simmental steers (10 or 40 mg Cu/kg DM, background 5.1) (Engle and Spears, 2001), in feedlot Brangus bulls (40 mg Cu/kg DM, background 5.8) (Netto et al., 2014) and in beef heifers (16.8 and 98 mg Cu/kg DM complete diet) (Alvarado-Gilis et al., 2014).

The effect of copper on the composition of dairy cows' milk has been less studied and the changes in lipid metabolism are scarce. Increasing the dietary concentration of copper sulphate (40 mg/kg DM, background 8.9) was associated with a decrease in milk polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) (trans-C18:1 and conjugated C18:2) concentrations in the study of Engle et al. (2001), although the effect was small. Similarly, Morales et al. (2000) found in copper-depleted cows increased C4:0, trans-C18:1 and conjugated C18:2, and decreased C16:1 in milk. In the study of Sinclair et al. (2013), milk PUFA content was unaffected by the dietary copper source (10 mg Cu/kg DM as copper sulphate or chelate; background copper 6.8 mg/kg) despite the decrease in milk C18:3 observed in cows when fed organic copper and that the inclusion of antagonists (sulfur, molybdenum) decreased C17:0 and C18:0 milk concentrations.

The effect of copper supplementation on lipid metabolism has been less studied in other ruminant species as compared to bovines. Cheng et al. (2010) analysed the effect of copper supplementation (10 or 20 mg Cu/kg DM; background copper 6.7 mg/kg) on hormones and enzymes which affect lipid metabolism in sheep; the reduction of backfat thickness may be due to reducing synthesis of adipose tissue and increasing lipolysis of adipose tissue. Senthilkumar et al. (2008) predicted 16 mg Cu/kg DM as the optimum copper concentration required in the diet for higher humoral immune response in Nellore Brown lambs on the basis of the results of their experiment with the supplementation of 7 or 14 mg Cu/kg to a basal diet (7.4 mg Cu/kg). Copper supplementation of feed of 1.5-year-old goats (supplement: 19 mg Cu/kg DM; background: 4.7) had an effect on lipid metabolism (decreased plasma cholesterol) (Zhang et al., 2012). Huang et al. (2013, 2014) found, in their studies with Jianyang Big-ear goat kids fed diets supplemented with 0, 20, 40, 80, 160, 320 and 640 mg Cu/kg DM

[10] End points measured: blood and liver concentrations of antioxidants (α-tocopherol, ascorbic acid, vitamin A, superoxide dismutase, glutathione peroxidase), prooxidants (Cu), concentrations of lipids (triglycerides and cholesterol), fatty acid composition, thiobarbituric acid-reactive substances (TBARS), and clinical chemical (creatine kinase and glutamate-oxaloacetatetransaminase) and haematological variables that indicate the level of oxidative stress.

(background copper 14.3 mg/kg), that dietary copper did not influence serum lipid profile, whereas it increased backfat depth, intramuscular fat and percentage of PUFA in M. longissimus dorsi but decreased longissimus muscle area; the findings on meat quality (colour reflectance spectroscopy, shear force) were ambiguous.

In conclusion, there might be some effects of increased dietary copper on lipid metabolism in ruminants; however, the results are inconsistent. Interactions between copper and lipid metabolism in ruminants are complex and poorly understood.

3.3. Copper in feedingstuffs

Data on copper in feedingstuffs are reviewed below in a condensed form. Details are shown in Annex B (Tables B.1, B.2, B.3 and B.4) for the copper content of feed materials as well as in Annex C (Table C.1) on the background concentrations of copper in complete feed. Appendices C and D include data collected from European countries concerning monitoring of copper in feed.

3.3.1. Copper in feed materials

Copper concentrations in plants and plant materials are influenced by the geological origin of soil, namely by copper concentration in soil, soil conditions (e.g. pH, ion exchange capacity) which influence copper uptake, fertilisation (e.g. (pig) manure), application of copper as fungicide (e.g. viniculture) and genetic differences in plant species, part of plant, stages of maturity, etc. Due to plant processing (milling, extraction processes, etc.) element concentration can be altered (e.g. concentration, contamination, dilution). Copper concentrations in plant materials are in the range 1–75 mg/kg; cereals, legumes and oilseeds contains 5–35 mg/kg, oilseed meals between 5 and 60 mg/kg and forages contain between 3 and 10 mg Cu/kg DM (Annex B, Tables B.1 and B.2). These figures are in line with recent German analyses of cereals and forages (Tables D.3 and D.4). Feed materials of animal origin (meals of bone, meat, feathers) contain high copper levels (15–50 mg Cu/kg). The copper concentration in fish meal ranges from 5 to 10 mg Cu/kg, except for fish solubles, where the concentration amounts up to 36 mg/kg (Appendix B, Tables B.1 and B.2).

Large differences in copper content were found in the literature for sugarcane molasses: 6–9 mg/kg (CVB, 2009), 29 mg/kg (INRA, 2004) and 66 mg/kg (NRC, 2000), and for distillers grains and solubles: 5 mg/kg (CVB, 2009),[11] 10 mg/kg (INRA, 2004) and 10–84 mg/kg (NRC, 2000). The higher values (e.g. 84 mg/kg) are typical of dark grains originating from whisky production in copper vessels and stills.

The highest copper content in feed materials has been found in dried grape pomace (grape marc) amounting to 75 mg Cu/kg (INRA, 2004; see Appendix B, Table B.2). FEEDIPEDIA (a database jointly managed by INRA, CIRAD, AFZ and FAO),[12] reports a range between 80 and 134 mg Cu/kg (91% DM) for grape pomace and between 65 and 160 mg Cu/kg (92% DM) for grape pulp (data collected during 1976–2002). More recent data (Spanghero et al., 2009) showed values between 102 and 124 mg Cu/kg DM for Italian grape pulp (seeds excluded), while for Californian pulp the copper content amounted only to 23 mg/kg DM. Also, Spanish fermented grape and olive marc, used as feed material, showed a copper range between 80 and 95 mg/kg (25% DM) (Salgado et al., 2015). Most probably this is related to the use in Europe, for more than one century, of copper-containing fungicides in vineyards and olive orchards. In the EU, a maximum residue limit (MRL) for grapes is set at 50 mg Cu/kg (EC 1410/2003)[13].

Regarding the well-known interactions between copper–molybdenum–sulfur–iron, especially in ruminants, it should be mentioned that the mean concentration of molybdenum in feed materials is normally below 1 mg Mo/kg, except for oilseed meals (2–4 mg Mo/kg) and lucerne and grass (1.5–3 mg Mo/kg), and for total mixed ration (TMR) below 1.5 mg Mo/kg DM (Van Paemel et al., 2010). However, depending on the type of soil (e.g. peats, calcareous sands), concentrations of molybdenum may be up to 7 mg Mo/kg in grasses and 20 mg Mo/kg in legumes (Kabada-Pendias, 2011). The iron content in TMR ranges between 200 and 400 mg/kg (Van Paemel et al., 2010). The sulfur content in cereals and oilseed meals ranges between 1 and 5 g/kg, whereas in roughage feeds it mostly varies

[11] A more recent publication of the CVB database (2011) showed only minor differences in copper content of feed materials.
[12] http://www.feedipedia.org/
[13] Regulation (EC) no 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. OJ L 70, 16.3.2005, p. 1.

between 1.0 and 10 g S/kg DM, of which up to 50% originates from SO_4^{2-} (Van den Top, 2005; Kamphues et al., 2014).

3.3.2. Copper in complete feed: background levels

Background levels are defined as the trace element concentrations in the complete feedingstuffs delivered by the feed materials. Hence, a background level simulation implies combining data of trace element composition tables of feed materials with complete feedingstuff composition data. The copper background levels were calculated for a list of animal species/categories complete feed formulations (based on crude nutrient concentrations of feed materials from CVB (2007); n = 35; Annex C, Table C.1) using the data for copper from CVB (2009) or INRA (2004) and Batal and Dale (2008).[14] Table C.1 does not include copper from trace element premixtures but includes the copper element concentrations for mineral sources (considered as feed materials), according to the data from Batal and Dale (2008).

Differences between the two simulated background level values for the same complete feedingstuff formulations are mainly due to differences in copper content in the feed materials data from CVB (2009) and INRA (2004) tables. More data are available on copper content in feed materials in the CVB tables than in the INRA tables. In order to have the same amount of feed materials in both simulations, for feed materials for which no copper content was available in the INRA tables, CVB values were used to complete the data set.

From Table C.1 (Annex C), it becomes evident that, for food-producing animals and dogs/cats, the copper background levels in complete feeds are rather low and in the range of 5 (salmon feed) to 15 (rabbit feed) mg Cu/kg feed. Higher values were noted for dairy cows mineral feed containing about 20 mg Cu/kg feed.

3.3.3. Feed additives

Several compounds of copper are currently authorised as nutritional feed additives in the EU: cupric acetate, monohydrate; basic cupric carbonate, monohydrate; cupric chloride, dihydrate; cupric methionate; cupric oxide; cupric sulphate, pentahydrate; cupric chelate of amino acid hydrate; copperlysine sulphate,[5] cupric chelate of glycine hydrate,[15] copper chelate of hydroxy analogue of methionine,[16] dicopper chloride trihydroxide (TBCC)[17] and copper bislysinate.[18]

3.3.4. Copper content in compound feed

EFSA launched a call for data throughout the EFSA's Focal Points to collect data on the official feed control on copper monitoring. In total, data from 14 European countries[19] were received, covering a total of 9,992 feed samples; the bulk of the data refers to feed for pigs, poultry and ruminants, followed by feed for horses, rabbits, fish, dogs and cats. The raw data were submitted to a validation

[14] The copper content in feed materials in CVB (2011) does not differ essentially from the data present in CVB (2009), which is used in the current opinion. Other sources consulted, but not further considered, include Atlas Premier 2014 (UK) and the online FEEDIPEDIA database.
The copper content of feed materials reported in Atlas Premier 2014 (UK) was not used for the following reasons: (i) less complete database compared to CVB (2009) and INRA (2004); (ii) the origin of the data is not clear; the editors refer to INRA tables (2002) and state that the analytical data has a UK bias; and (iii) several other weak points were noted: no data on silages/roughages/forages and no differences were noted in copper content of e.g. oilseeds expeller vs extracted or within animal products (meat meal vs meat and bone meal), which is not a normal phenomenon.
The data from FEEDIPEDIA are not used as (i) Feedipedia is a joint project of the Institut National de la Recherche Agronomique (INRA, French National Institute for Agricultural Research), the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD, French Agricultural Research Center for International Development), the Association Française de Zootechnie (AFZ, French Association for Animal Production) and the Food and Agriculture Organization of the United Nations (FAO), reporting feed materials originating mostly from tropical, subtropical and Mediterranean regions; (ii) it is very well known that chemical composition and nutritive values of a given feed material when grown in temperate and tropical region may differ substantially.

[15] Commission Regulation (EC) No 479/2006 of 23 March 2006 as regards the authorisation of certain additives belonging to the group compounds of trace elements. OJ L 86, 24.3.2006, p. 4.

[16] Commission Regulation (EU) No 349/2010 of 23 April 2010 concerning the authorisation of copper chelate of hydroxy analogue of methionine as a feed additive for all animal species. OJ L 104, 24.4.2010, p. 31.

[17] Commission Implementing Regulation (EU) No 269/2012 of 26 March 2012 concerning the authorisation of dicopper chloride trihydroxide as feed additive for all animal species. OJ L 89, 27.3.2012, p. 3.

[18] Commission Implementing Regulation (EU) No 12230/2014 of 17 November 2014. OJ L 331, 18.11.2014, p. 18.

[19] Belgium, Cyprus, Czech Republic, Estonia, Germany, Greece, Hungary, Ireland, Italy, Luxembourg, Portugal, Slovakia, Slovenia and the United Kingdom.

procedure (see Table C.1 in Appendix C). The remaining total of 5,806 samples was submitted to descriptive statistical analysis. The results are summarised in Table 8; for more details, see Appendix D (Tables and Figures D.1–D.17).

Table 8: Copper in compound feed: descriptive statistics of the control data submitted by 14 European countries

Animal group	Category[a]	n	mg Cu/kg complete feed			Samples above CAMC[b] (%)
			Median	10th percentile	90th percentile	
Poultry	Starter chicks	63	14	10	25	7.9
	Laying hens	440	15	9	23	5.9
	Chicken for fattening	360	19	12	24	7.2
	Ducks/geese for fattening	91	17	12	23	4.4
	Turkeys for fattening	203	18	14	24	7.9
Pig	Piglets	1,420	136	23	168	8.8
	Pigs for fattening	2,034	18	12	26	12.9
	Sows	546	20	13	29	20.1
Bovid	Calves milk replacer	111	8	4	13	3.6
	Fattening cattle	42	12			0.0
	Dairy cow	31	13			6.5
	Sheep	30	8			0.0
	Goat	8	9			0.0
Horse		2	30			
Rabbit		202	19	14	25	10.4
Fish		40	13			0.0
Dog		131	14	10	23	6.9
Cat		53	17	12	21	1.9

(a): Where applicable, the following grouping was applied:
'Laying hens': Includes the data labelled as feed for Laying hens, Layer Phase I and Layer Phase II.
'Chickens for fattening': Includes the data labelled as feed for Chickens for fattening, Broiler starter, Grower and Finisher.
'Turkeys': Includes the data labelled as feed for Turkeys for fattening, Starter, Grower and Finisher.
'Piglets': Includes the data labelled as feed for Piglets weaned, Piglets starter I and Piglets starter II.
'Pigs for fattening': Includes the data labelled as feed for Pigs for fattening, Pig grower and Pig finisher.
'Sows': Includes the data labelled as feed for Breeding pigs, Sows, Sows gestating and Sows lactating.
'Rabbit': Includes the data labelled as feed for Rabbit, Rabbit breeder and Rabbit grower/finisher.
(b): CAMC: currently authorised total maximum content of copper in complete feed.

The FEEDAP Panel notices that the information provided by Table 8 may be limited as only 14 MS submitted data. However, the data show that, with the exception of complete feed for pigs for fattening and sows, the 90th percentile of dietary copper concentration is below the CAMC. The 90th of copper levels on feed for fattening pigs and sows is marginally above the CAMC, about 13% and 20% of all samples exceeded the CAMC.

Data for salmonid fish feed provided by the Norwegian Seafood Safety Authority Annual monitoring programme (Appendix D, Table D.18) showed a rather constant mean copper content of feed at about 10 mg/kg. The range over the years shows minimum and maximum levels between 2.5 and 65 mg Cu/kg, respectively.

3.4. Copper bioavailability and interactions/incompatibilities

3.4.1. Copper bioavailability in feed materials and additives

Until recently, the main focus was on the bioavailability of inorganic sources of copper. Cupric sulphate is often used as a standard, although cupric chloride, TBCC, cupric oxide, cupric carbonate and cupric acetate have also been extensively tested and are available commercially. However, over the last few years two other issues have refuelled interest in copper source and bioavailability. Firstly, the interest raised in organic sources of copper, which are chelated with amino acids, peptides or proteins. Secondly, and the most recent area of interest, is the consideration of the availability of trace

elements, such as copper, naturally present in feed materials. By tradition, requirements for copper and other trace elements are met entirely by supplements without taking into account the background levels. This recent interest stems from attempts at minimising the nutrient supply of copper and other minerals as a means of reducing excreta levels and so aiding environmental initiatives. While it is easy to measure total levels of copper in major ingredients, ascertaining bioavailability is a complex procedure (Leeson, 2009).

Copper absorption/availability of feedstuffs depends on the origin and may be affected by the presence of phytate, as well as phytase, in cereals and oilseeds but the magnitude of this in combination with the presence of other minerals and trace elements is not fully understood.

3.4.1.1. Poultry

A typical starter diet for chicken for fattening provides around 150% of the requirement for copper relative to NRC (1994) recommendations. The proportion that is available to the bird is predictable, although values can be quite variable. Such variation may well be impacted by agronomic conditions for cereals and vegetable proteins, and by prior nutritional status and processing conditions for animal proteins. As for any nutrient, bioavailability will be affected by parameters used in estimation, which for copper seem to range from simple production characteristics throughout to liver and bile accumulation indices.

Aw-Yong et al. (1983) suggested bioavailability values for copper in wheat, maize and barley to be around 80%. Using a bile accumulation index, Aoyagi et al. (1993) showed much lower levels of the relative copper availability in vegetable proteins; values being around 40% in soybean meal, cotton seed meal and groundnut meal and closer to 50% in corn gluten meal; these lower levels may be due to greater binding of copper with phytate. In a subsequent study, Aoyagi and Baker (1995) suggested that copper in soybean meal was only 40% as available (also using a bile accumulation index) as copper in cupric sulphate and that adding phytase to test diets decreased, rather than increased, copper availability. These authors ascribed the negative effect of phytase to the concomitant release of zinc, which negatively impacted copper uptake. More recently, Jongbloed and Thissen (2010) published a meta-analysis on quantification of the effect of microbial phytase on the bioavailability of copper and zinc in growing pigs and broilers; they concluded that, although the effect of microbial phytase on the digestibility of copper was significantly positive in broilers (increase in copper digestibility from 0% to 20%), no reduction of the addition of copper could be advised using microbial phytase in broiler diets, mainly owing to the low number of experiments (n = 3). Copper in animal proteins seems to be even more variable in bioavailability. It is well known that copper in pig liver is totally unavailable for poultry. Aoyagi et al. (1995) found that disrupting the protein structure in porcine liver increased bioavailability, with values of 32%, 46% and 63% seen after autoclaving, acid hydrolysis or protease digestion, respectively. In testing other animal proteins, Aoyagi et al. (1995) showed zero bioavailability in feather meal and only 4% in meat and bone meal manufactured from ruminants. The bioavailability of copper from pork meal and poultry by-product meal was 50% and 40%, respectively.

There has been a wealth of information published on bioavailability of inorganic sources and more recently of organic sources of copper, usually by comparison with cupric sulphate, using a range of assessment parameters (Jongbloed et al., 2002). Considering chick weight as well as liver copper levels and haemoglobin status, McNaughton et al. (1974) suggested that both cuprous oxide and cuprous iodide were 60% as bioavailable as sulphate. Pesti and Bakalli (1996) and Ewing et al. (1998) suggested copper citrate to be more efficacious than copper sulphate. Ledoux et al. (1991) established the relative bioavailability of cupric oxide, cupric carbonate and cupric sulphate, using supplementation levels of 150, 300 and 450 mg Cu/kg feed, based on copper levels in liver and setting cupric acetate at 100; the relative biological availability was estimated to be 88.5%, 54.3% and 0% for the sulphate, carbonate and oxide, respectively. Using copper acetate as a standard, Zanetti et al. (1991) found carbonate salt to be only 66% as available (measured as liver copper concentration). Feeding graded levels up to 390 mg Cu/mg diet, Liu et al. (2005) suggested TBCC to have a relative value of 134% compared to cupric sulphate, taking egg weight as the main criteria. Miles et al. (1998) proposed a value of 112% (based on liver copper deposition) for TBCC relative to sulphate. Also in terms of liver copper accumulation, TBCC was 109% as efficacious as sulphate (Luo et al., 2005); these authors also suggested that TBCC was less destructive to vitamins in both the feed and within the bird: liver and plasma vitamin E levels were higher in birds fed TBCC vs sulphate using a diet initially containing 36 IU/kg. Interestingly after storing feed for 21 days there was 16% loss of potency of tocopherol when TBCC was used, compared to 40% loss in the feed containing copper sulphate. TBCC and cupric sulphate (220 and 180 mg Cu/kg feed, respectively) equally improved carcass weight of chickens for

fattening after 45 days of feeding, compared to the negative control (30 mg Cu/kg feed) (Arias and Koutsos, 2006).

Recently, research interest has been focused on testing the efficacy of organic copper additives, including copper chelates with amino acids or proteins. It is often claimed that these chelates are preferentially absorbed and metabolised, and provide less chance for antagonism of copper with other minerals or substrates (Leeson and Summers, 2005); this latter assumption is obviously predicated on these copper sources remaining chelated until the site of digestion and absorption. Baker et al. (1991) ascribed a value of 115% to availability of copper from a copper–lysine complex compared to cupric sulphate, although the basal diet used in this study had an uncharacteristically high level of copper (290 mg Cu/kg diet). It is also claimed that organic copper sources are more effective than cupric sulphate in decreasing plasma cholesterol in broilers (Mondal et al., 2007b). Miles et al. (2003) found a copper amino acid chelate to be slightly less efficacious than cupric sulphate. Guo et al. (2001) investigated the solubility of different copper compounds in various solutions for their ability to predict copper availability in poultry measured as liver copper deposition; the authors found that solubility at pH 2 was the best predictor. This chicken study showed a somewhat better bioavailability of Cu-Lys and copper amino acid chelate, compared to copper sulphate.

There have been reports of very low levels of organic minerals being adequate for poultry, with a view to reducing mineral excreta output. Nollet et al. (2007) showed that broilers fed very low levels of all trace minerals in the form of proteinates performed as well as birds fed conventional levels as inorganic salts. Likewise, Bao et al. (2007) suggest that 4 mg copper (as cupric chelate of amino acid hydrate)/kg diet (and 40 mg/kg each of iron, manganese and zinc) is adequate for birds. In such studies it is important to realise that all minerals are at low levels so as not to induce antagonisms. Feeding low levels of copper in combination with regular levels of other trace minerals, and especially zinc, will likely result in a greater chance of inducing copper deficiency. More recently, Jegede et al. (2011) reported significant higher daily weight gain in broilers fed copper proteinate compared to cupric sulphate supplemented at 50–150 mg Cu/kg feed for 56 days (background 35 mg Cu/kg feed).

It can be concluded that copper in plant- and animal-derived feed materials has a relative bioavailability of less than 50%. Compared to cupric sulphate as the reference standard copper source, cupric carbonate, cupric oxide and cuprous iodide seem to have a lower relative bioavailability in poultry, whereas TBCC, cuprous oxide and organic copper sources are, at least, as available as cupric sulphate. Effects of phytase addition are rarely examined in poultry and the few results that are available show rather a small and inconsistent impact on copper absorption.

3.4.1.2. Pigs

Apparent total tract digestibility (ATTD) of copper in a maize/soybean meal compound feed (4.2–5 mg Cu/kg) for pigs ranged between 7.6% and 15.9% (Adeola, 1995) and between 31.8% and 55.4% (Liu et al., 2014a). Supplementation of 10–35 mg Cu/kg diet (from copper sulphate) to a maize/soybean meal diet resulted in an ATTD of copper that ranged between 11.9% and 24.2% (Apgar and Kornegay, 1996; Wang et al., 2005; Zacharias et al., 2007).

The antinutritional effects of phytic acid in animal nutrition are well known (Pallauf and Rimbach, 1997), and the presence of phytate in cereal grains and oilseeds may depress the availability of copper and other trace elements in monogastric animals and humans (Morris and Ellis, 1989). Phytate is negatively charged and may bind positively charged ions such as Ca(II), Zn(II) and Cu(II) rendering the minerals less available. As such, phytic acid has been shown to reduce the apparent mineral and trace element digestibility in pigs and poultry as recently reviewed by Woyengo and Nyachoti (2013), although most studies on phytic acid/phytase are carried out with pigs. Inconsistent results were found by Adeola (1995) regarding the effect of phytase addition to pig diets on copper digestion: with diets, not supplemented with copper (background 5 mg Cu/kg diet) there was a small increase in the ATTD of copper (from 7.6% to 15.9%), while with diets supplemented with 60 or 120 mg Cu/kg diet, no increase in ATTD of copper was noted due to phytase addition. Kies et al. (2006) noted a small change in ATTD of copper of diets containing 113 mg Cu/kg when phytase was added (from −5.8% to 6.0%). Madrid et al. (2013) reported a change in ATTD of copper (from −0.8% to 34.3%) when phytase was added to a diet containing 18.5 mg Cu/kg, while copper concentration in liver and muscle was not affected. A recent meta-analysis on the effect of phytase on phytate hydrolysis and mineral availability in pigs showed effects of microbial phytase addition on the digestibility of zinc but not of copper (Bikker et al., 2012a). Consequently, no consistent effect of microbial phytase on copper status (serum, liver) of pigs could be detected when phytase was added to copper-supplemented as well as copper-unsupplemented diets (Bikker et al., 2012b). This inconsistency on reported effects of microbial

phytase on copper may be due to antagonistic interactions with e.g. zinc as reported in chicken (Aoyagi and Baker, 1995). No data are available on the effects of endogenous phytase present in some non-heat-treated cereals and feedstuffs.

The bioavailability of copper may differ among different copper salts, and for many years has generally been accepted that the bioavailability of copper is superior in cupric sulphate compared to cupric sulfide and cupric oxide (Cromwell et al., 1998; NRC, 2012). As such, cupric sulphate has been widely used as a source of extra copper. Although Baker (1999) stated that cupric oxide (bioavailability = 0 compared to cupric sulphate = 100) should not be used as copper supplement in animal nutrition, large variation in bioavailability has been reported for this copper source. Bunch et al. (1961) did not find any difference in growth performance of piglets receiving 250 mg Cu/kg from cupric sulphate or cupric oxide whereas, in the trials of Cromwell et al. (1989), cupric oxide in contrast to cupric sulphate totally failed to influence performance or copper contents in the liver at dietary supplementations between 125 and 500 mg Cu/kg feed. Corresponding differences were obtained in experiments with sows fed either cupric sulphate or cupric oxide (see Section 3.2.3). Differences in manufacturing processes of the copper salts seem to be the cause as the use of an ammoniacal leach process results in a highly soluble form of copper, while a process using surface heating and milling of metallic copper yields rather insoluble forms. More recently, it became clear that the bioavailability of copper from cuprous oxide (Cu_2O) does not essentially differ from that of cupric sulphate (Baker, 1999; Leeson, 2009).

The availability of copper from organic chelates (e.g. Cu-Lys, Cu-Met) and other complexes of copper (e.g. TBCC) has been shown to be comparable to copper sulphate (e.g. Apgar et al., 1995; Apgar and Kornegay, 1996; Jongbloed et al., 2002; Shelton et al., 2011; NRC, 2012) although the growth-promoting effect seems to be greater in pigs fed the organic complexed substance compared to cupric sulphate (Coffey et al., 1994; Zhou et al., 1994a; Jondreville et al., 2002; NRC, 2012).

In conclusion, the apparent absorption of intrinsic copper in maize/soybean meal diets ranges between 10% and 50%. Adding cupric sulphate decreased these values to 10–25%. No consistent positive effects of adding phytase on copper absorption/bioavailability could be derived from the literature. The bioavailability of cupric carbonate and cupric oxide is very variable and depends on the manufacturing process. TBCC, cuprous oxide and organic chelates of copper showed in pigs essentially an equal availability as cupric sulphate.

3.4.1.3. Other monogastric species

Apart from pig and poultry, copper availability from feed materials and additives has been scarcely studied in other monogastric species. In general, it is assumed that organic forms are more bioavailable compared to inorganic (copper sulphate being the reference). In horses, the apparent copper absorption and retention (as a percentage of intake) were greater for animals receiving Cu-Lys chelate compared to those fed the inorganic copper sulphate (Wagner et al., 2011). Similarly, significantly higher blood copper concentrations were found in mares receiving copper in an organic (copper proteinate) compared with an inorganic (copper sulphate) form (Jančíková et al., 2012). In dogs, it was observed that copper in chelated forms is more readily available to bitches compared with the inorganic forms (Kuhlman and Rompala, 1988). On the contrary, Cavalcante et al. (2002) did not find any difference in copper bioavailability (hepatic copper deposition) between organic and inorganic sources of copper (oxide, sulphate, carbonate and a chelate) in rabbits.

Although cupric oxide is available in ruminants (see *Ruminants* in next paragraph), it has a low availability in many other species (Baker and Ammerman, 1995). It was completely unavailable in cats (Fascetti et al., 1998) and, because of its poor bioavailability, the use of cupric oxide as a nutritional source is excluded for dogs (FEDIAF, 2012).

3.4.1.4. Ruminants

Little is known about the forms of copper in feeds, but it is assumed that copper absorption from different feeds is determined by the synchronicity of the release of copper and its potential antagonists (mainly sulfur, molybdenum, iron) in the rumen. Fresh grass is a poor copper source whereas brassicas, cereals and certain cereals by-products are good sources of copper for sheep (Suttle, 2010). The absorbability of copper in natural foodstuffs of low molybdenum content (< 2 mg/kg DM) in Scottish ewes was estimated in the range of 1.4–12.8% (grazed herbage – July 2.5 ± 1.1%; grazed herbage – September/October 1.4 ± 0.9%; silage 4.9 ± 3.2%; hay 7.3 ± 1.8%; cereals 9.1 ± 1.0%; leafy brassicas 12.8 ± 3.2%) (Suttle, 2010). Van den Top (2005) calculated true copper absorption from examples of ruminant rations based on molybdenum and sulfur concentrations in the Netherlands:

dry cow 3.9%, lactating cow 5.8%, dry sheep/goats 5.4%, lactating goat 7.4% and growing lambs 3.6%.

The bioavailability of copper from different sources is influenced by their solubility in the gastrointestinal apparatus and mainly in the rumen. Trace mineral solubility can greatly affect the total concentration that is available for rumen microbes because only soluble minerals are available for use or interactions. Copper sulphate is the standard source to which other copper sources are compared. Copper oxide is relatively insoluble even under acidic conditions, and in the form of powder passes through the acidic environment in the abomasum before it is solubilised (Spears, 2003). The TBCC (Cu_2OH_3Cl) is less soluble in the rumen than copper sulphate, although both forms have similar solubility in the abomasum, suggesting that this mineral from both forms should be available for absorption in the intestine, but chloride forms lower amounts of complexes with sulfur and molybdenum in the rumen (Genther and Hansen, 2015). TBCC chloride and copper sulphate had similar bioavailability in beef cattle and heifers fed corn- and molasses-based supplements (Arthington et al., 2003; Arthington and Spears, 2007), but was more available (based on liver copper 196%) when supplemented to diets high in molybdenum and sulfur (Spears et al., 2004). Copper carbonate had lower solubility in water but under acidic conditions was soluble (74%); however, it did not increase copper in the liver in comparison with copper sulphate even in the diet high in S and Mo (Ward et al., 1996). Ledoux et al. (1995) estimated the following relative copper bioavailability of inorganic copper compounds in sheep: chloride 100%, acetate 93%, sulphate 142%, carbonate 121% and oxide 35%.

Several organic forms of copper have been tested: copper proteinate, copper lysine, copper glycine, copper methionine or some other copper chelates. Considerable variation was described between different copper sources with respect to their bioavailability. In some studies the efficiency of absorption of copper proteinates exceeded that of cupric sulphate; however, in most studies copper proteinates have nearly the same efficiency of absorption as copper sulphate. Higher bioavailability of organic copper compounds (glycinate, proteinate, chelate) was not mostly found when the diet did not contain antagonists (Du et al., 1996; Ward et al., 1996; Engle and Spears, 2000a; Cheng et al., 2008; Sinclair et al., 2013). Nevertheless, some authors found higher bioavailability of Cu-Met also when the diet without antagonists was fed (Sanjivani et al., 2014). Pal et al. (2010) found in ewes a gut absorption of copper sulphate of 21.9% and of copper methionine of 33.1%; the bioavailability was 100% and 141%, respectively, based on liver copper. Jongbloed et al. (2002) reviewed the relative copper compound bioavailability for ruminants on the basis of copper liver concentration: copper sulphate 100%, copper carbonate 93%, copper oxide 76%, Cu-Met 91% and Cu-Lys 104%. However, Pott et al. (1994) reported a relative copper bioavailability of Cu-Lys for lambs of 68% compared with copper sulphate.

It is assumed that higher bioavailability of organic forms is due to lower formation of unsoluble complexes (thiomolybdates). The solubility of copper proteinate and copper chelate in water was 75% and 10% and in acidic environment 99% and 87%, respectively (Ward et al., 1996). The different solubility of organic compounds could explain some differences in the experimental results. Higher bioavailability in comparison with copper sulphate was found when feeding diets with high molybdenum (6.0–6.9 mg/kg DM) and sulfur (2.3–3.9 g/kg) in copper chelate (Ward et al., 1996) and copper glycinate (Hansen et al., 2008); however, another experiment with high molybdenum (8.9 mg/kg) and sulfur (3.5 g/kg DM) showed similar bioavailability like copper sulphate in copper proteinate (Sinclair et al., 2013). Van den Top (2005) summarised the results of bioavailability trials with different copper sources for ruminants and concluded that the evidence is not fully in agreement. In the presence of low molybdenum concentrations in the ration (concentration less than approximately 2 mg/kg DM) the differences in bioavailability between the different copper sources seem to be minor; however, in the presence of higher molybdenum concentrations (± 5–7 mg/kg DM), copper proteinate may be more advantageous.

3.4.1.5. Fish

Read et al. (2014) investigated the effects of different dietary protein- (fishmeal vs plant-based: soy- and corn protein concentrates) and copper sources (copper sulphate vs copper-lysine) in rainbow trout. Fish were fed plant-based diets supplemented with copper sulphate or copper lysine at 0, 5, 10, 15 or 20 mg/kg or fishmeal-based diets supplemented with copper sulphate or copper lysine at 0, 5, 10 or 20 mg/kg for 12 weeks. Copper concentration in the diet did not affect weight gain. FCR was significantly lower in trout fed plant-based diets supplemented with 10 or 15 mg/kg compared to other groups, whereas there was no significant effect of copper level on FCR in fish fed fishmeal-based diets. No differences were found in growth rates or liver copper concentrations in fish fed diets

supplemented with either copper sulphate or copper lysine. Fish fed plant-based diets had significantly higher weight gain, lower FCR, and higher liver copper concentrations than fish fed the fishmeal-based diets for 12 weeks.

Prabhu et al. (2014) calculated the relative efficiency of amino acid chelates of copper compared to copper sulphate, based on whole-body or tissue mineral retention as the criteria, and did not find significant differences.

3.4.1.6. Crustaceans

Zhou et al. (2014) compared copper sulphate and TBCC as dietary copper sources in Pacific white shrimp (*L. vannamei*). Diets were supplemented with 6, 12 and 24 mg Cu/kg either with copper sulphate (analysed values 30.0, 30.4 and 48.4 mg Cu/kg DM) or the same levels of TBCC (analysed values 28.3, 29.8 and 48.7 mg Cu/kg DM), and fed to shrimp for 8 weeks. There were no significant effects of copper source or dietary concentration on final mean weight, final biomass, FCR or survival. The source of copper had a significant effect on tissue levels, indicating a lower bioavailability of TBCC in Pacific white shrimp.

3.4.2. Interactions with dietary constituents

Interactions involving copper may take place in several steps during the overall metabolism of copper from the feed intake to the achievement of the active physiological function of copper in the body of animals. However, the importance and extent of interactions – synergistic as well as antagonistic – may depend on species, diet composition, feedstuffs, locations for crop growing (soil conditions), seasonal factors, crop genetics, etc. (Suttle, 2010; Cano-Sancho et al., 2014). Interactions may affect the absorption of copper (Section 3.1.2) and thus, at which dietary copper supply, deficiency (Section 3.2.2), adequacy (Section 3.2.1) as well as toxicity (Section 3.2.3) can be seen. As such, interactions may contribute to the variation in reported effects of copper on physiological and growth responses described in the literature and thereby contribute to the difficulties in the establishment of an understanding of the basic nature of copper effects.

Interactions may occur in the gastrointestinal tract involving other minerals or other dietary components. Often, the effects of dietary copper are studied together with zinc as these metals are known to interact at different points starting in the gastrointestinal tract as both metals can participate in the formation of phytate complexes whereby copper and/or zinc (and other cations) can be precipitated affecting the availability of the metals (Section 3.4). Further, copper and zinc may interact during the processes of intestinal uptake and transfer involving MT that coats the surface of the luminal side of the intestinal cells and binds and sequesters copper at the luminal surface (Section 3.1.2). Therefore, zinc is a potent inhibitor of copper absorption as seen in e.g. horses (Cymbaluk and Smart, 1993) and other monogastrics (Cousins, 1985; Davis and Mertz, 1987; Suttle, 2010). Copper and iron are also well known to interact and both metals are required for haematopoiesis (Section 3.2.2). As such, deficient supply of one the metals results in anaemia. The interaction between copper–sulfur–molybdenum is solely related, but very important, to ruminants and will later described in detail in this scientific opinion.

3.4.2.1. Interactions with other trace minerals in monogastric animals

Many interactions between copper and other minerals have been detected and may be of nutritional importance in monogastric animals (see above). Thus, specific well-defined interactions with copper could protect against deficiency or toxicity when diets are not suitable balanced with other nutrients as reported by Suttle, 2010 (will not be addressed further). The presence of phytate in cereal grains and seeds may also affect the magnitude of interactions between copper and other trace elements in monogastric animals as phytate is negatively charged and may bind positively charged ions as Ca(II), Zn(II), Cu(II), etc., rendering the minerals less available for absorption or interactions in the gastrointestinal tract (Section 3.4.1).

As mentioned in the Introduction to this chapter, copper and zinc interactions are mutual and zinc may inhibit absorption of copper and vice versa copper may inhibit zinc uptake (EFSA FEEDAP Panel, 2014). In piglets, dietary levels of e.g. 3,000 mg zinc (as oxide)/kg diet are supplied to newly weaned piglets. As such, there might be, according to NRC (2001), a risk of detrimental interactions between copper and zinc that may interfere with the absorption of copper. Hill et al. (2000) found in a study with weaned piglets (6.55 kg; average 22.2 days) supplemented with 3,000 mg Zn (as ZnO)/kg diet and 250 mg Cu (as sulphate)/kg diet, fed alone or in combination, that both minerals stimulated

growth but the combination of zinc and copper did not result in an additive growth response. As a result, Hill et al. (2000) suggested that the tradition of adding high copper levels should be discontinued and that newly weaned piglets could benefit from 'pharmacological' zinc levels. According to Carlson et al. (2007), interactions between zinc and copper resulted in increased plasma zinc level when 175 compared to 20 mg Cu/kg diet was fed to weaned piglets irrespective of dietary zinc level (100 or 2,500 mg/kg diet (as ZnO)). This indicates that copper fed above requirements may improve zinc status due to a synergetic effect on zinc absorption caused by the use of 'pharmacological levels' of dietary copper. At the same time, the increased dietary copper level (20 vs 175 mg Cu/kg diet) had no significant effect on plasma copper concentration.

Dietary interaction between copper and selenium has been reported in Atlantic salmon, with a positive correlation between liver copper and selenium concentration (Lorentzen et al., 1998). Dietary selenium was also found to reduce oxidative stress associated with elevated dietary copper (20 mg/kg) in grouper (Lin and Shiau, 2007) and in chicken (800 mg Cu/kg diet) (Suttle, 2010). Interactions between zinc and copper were observed for gastrointestinal uptake in rainbow trout, examined through *in vitro* gut sac technique (Ojo et al., 2009), where a high concentration of zinc reduced the absorption of copper and vice versa. However, no antagonistic effects of high dietary supply of zinc on copper or vice versa were observed in the utilisation of copper and zinc by rainbow trout from chelated or as inorganic copper sources (Read et al., 2014).

Rambeck et al. (1991) and Rothe et al. (1994) studied the effect of high copper supplementation to pig feed on the deposition of cadmium which was added to the feed as cadmium chloride in a quantity of 1 mg/kg. High dietary copper (175 and 200 mg/kg feed) increased cadmium retention in the kidney and to a lesser extent in the liver. The findings provide only weak evidence of an adverse effect of high dietary copper, but the data need confirmation under up-to-date experimental conditions.

In conclusion, the importance of interactions between copper and other minerals should be recognised irrespective of whether they are synergistic or antagonistic. The nature of interaction between, for example, copper and zinc may be either synergistic or antagonistic depending on the dietary supply of both minerals. This draws attention to the importance of a balanced dietary mineral supply (Suttle, 2010).

3.4.2.2. Interactions with other trace minerals in ruminants

Copper absorption in ruminants is low (< 1–10%) relative to values reported in non-ruminants (Spears, 2003). In newborn calves, up to 70% of dietary copper is absorbed; however, with the development of the rumen the absorption decreased and in adult cattle only 1–5% of dietary copper is absorbed (NRC, 2001). The absorption of copper of mature ruminants from the digestive apparatus is influenced mainly by the presence of antagonists such as sulfur, molybdenum and iron.

The microbial metabolism of inorganic and also organic sulfur compounds in the rumen results in the production of sulfide. Reactions occur between sulfur and molybdenum enabling the formation of thiomolybdates (mono-, di-, tri- and tetrathiomolybdates), which bind copper with a high affinity (mainly tri- and tetrathiomolybdates irreversibly bind copper) and create copper thiomolybdates which are not absorbed in the intestine, because copper associated with these compounds is insoluble. In the absence of rumen-available copper, all thiomolybdates can be absorbed rapidly through the rumen wall or more sedately via the small intestine, and thiomolybdates can bind to copper in biological compounds (Gould and Kendall, 2011). Thiomolybdates were detected in plasma and it is supposed that they increased biliary excretion of copper from liver stores and tetrathiomolybdates also accumulate in plasma as albumin-bound trichloroacetic acid-insoluble complexes with a much slower clearance rate than normal albumin-bound copper, which results in reduced transport of available copper for biochemical processes (Suttle, 2010). It was shown *in vitro* that tetrathiomolybdate inhibits Cu-containing oxidase enzymes (ceruloplasmin, cytochrome oxidase, superoxide dismutase, tyrosinase) in physiologically relevant concentrations. The inactivation of ceruloplasmin does not occur by removing copper from the compounds, but the tetrathiomolybdate binds to the ceruloplasmin through the sulfide groups, reducing Cu(II) to Cu(I) (Gould and Kendall, 2011). There is no consensus on whether the reaction of tetrathiomolybdates to copper compounds is irreversible or not; in this way, it may develop clinical problems of copper deficiency, even though it is a thiomolybdate toxicity.

For practical reasons the Cu:Mo ratio (mg/kg DM) is used to predict risk of copper deficiency. The interpretation is influenced by many factors, generally ratios < 1 indicate a high risk of copper deficiency and ratios > 3 are considered safe. The increase in molybdenum concentration from 1 to 5 mg/kg DM decreases the true copper absorption about 1% (Van den Top, 2005). The concentration

of molybdenum in the diet of ruminants is generally < 1.5 mg/kg DM; however, in some feed materials, the content is relatively high (alfalfa 1.4–2.2; soybean meal 3–4; peas 3 mg Mo/kg DM) (Van Paemel et al., 2010) and, for example, in grass the content varies from 0.9 to 5.4 mg Mo/kg DM, depending on the type of soil (Van den Top, 2005). Taking into account these values, the ratio of Cu:Mo in Europe is mostly > 3; however, a ratio of < 3 may be seen in small localised areas.

Independent of its role in Mo–Cu interaction, sulfur can reduce copper bioavailability also via the formation of insoluble copper sulfide (CuS, Cu_2S). In the ration, an MTL of 4 g S/kg DM is suggested (NRC, 2001); however, a depression of copper absorption starts from 1 g S/kg DM (Suttle, 2010). The increased amounts of sulfur from 0.12% to 0.31% or 0.46% DM decreased liver copper concentrations (from 230 to 140 or 96 mg Cu/kg DM, respectively) in steers (Spears et al., 2014) and similarly lower plasma copper was found (11.8 μmol/L vs 16.7 μmol/L) in steers feeding with 0.68% S in comparison with 0.24% S (Pogge et al., 2014). The amount of sulfur available for interaction with copper is influenced by the degradation of sulfur compounds by rumen microbes, the amounts of rumen degradable protein, fermentable carbohydrates and also feeding frequency, which affects ruminal pH (a rapid decrease in rumen pH gives higher production of sulfide). The concentration of sulfur in feeds mostly varies from 0.5 to 2 g/kg DM; however, higher values are found, for example, in grass (1.8–4.3 g/kg DM; Van den Top, 2005) or ethanol by-products (3–10 g/kg DM; Pogge et al., 2014). Variable amounts of sulfur are also present in water; when reference values for sulphate and sulfide are not exceeded, the intake of sulfur from drinking water is 8–13 g/day (Van den Top, 2005). It can be concluded that sulfur is present in rations in sufficient quantities for interactions and can markedly affect copper absorption.

The effect of sulfur and molybdenum varies depending on the feedstuff serving as a source of copper: in silages, molybdenum has a small effect, but sulfur reduces copper absorption in a logarithmic manner; in hays, the inhibitory effect of molybdenum and sulfur is relatively low; in fresh grass copper absorption is greatly reduced by small increments in molybdenum and sulfur contents and, similarly, both sulfur and molybdenum markedly affects copper absorption also in concentrate-type diets (Suttle, 2010).

The other element involved in Cu–S–Mo interactions, and can negatively influence copper absorption, is iron. Copper–iron interactions are more pronounced when high iron and high sulfur are present in the diet. In the rumen, iron can react with sulfide and copper either to produce the Fe–Cu–S complex, which reduces the amounts of copper available in the rumen for reaction with thiomolybdates, or to create iron sulfide. In the abomasum, sulfur is released from FeS to form insoluble CuS. Thus, the action of iron on copper absorption is, to some extent, independent of molybdenum. Besides this, ferric oxide can absorb copper and, in this way, reduces its absorption (Van den Top, 2005; Gould and Kendall, 2011). To roughly assess the effect of iron on copper the absorption Fe:Cu ratio can be used. Ratios > 100 indicate a high risk of copper deficiency and ratios < 50 are considered safe.

The concentration of iron in the diet of cattle varies between 200 and 400 mg Fe/kg DM, yet the content in individual feed materials varies considerably (lucerne 212–553; grass 149–443; wheat 57; rapeseed 82; rapeseed meal 499–534; mineral feeds-phosphates 7,000–15,000 mg Fe/kg DM) (Van Paemel et al., 2010) and, for example, in grass, iron content can be 110–1,400 mg Fe/kg DM according to the type of soil (Van den Top, 2005). The iron content of the diet, including dust or rain soil splash, can range from 50 to 4,000 mg Fe/kg DM (Van den Top, 2005). Taking into account these values, the antagonistic effect of dietary iron on copper absorption may be of practical importance.

Suttle (2010) proposed marginal bands for copper antagonist concentrations in the diet of ruminants to aid the diagnosis of copper responsive disorders (CRD) (Table 9). As nearer the proportions for Cu/Mo and Fe/Cu and the copper levels approach the bold value, the higher is the risk for CRD.

Table 9: Marginal bands for copper antagonist concentrations in the diet to aid the diagnosis of CRD in ruminants on fresh herbage or forage-based diets (from Suttle, 2010)

	Diet based on	Cattle and Sheep	Goats	Interpretive limits
Cu/Mo	Herbage	**1.0**–3.0	**0.5**–2.0	Diet S > 2 g/kg DM
	Forage	**0.5**–2.0	**0.3**–1.2	Diet Mo < 8 mg/kg DM for sheep, goats Diet Mo < 15 mg/kg DM for cattle
Fe/Cu	Herbage	50–**100**	50–**100**	

	Diet based on	Cattle and Sheep	Goats	Interpretive limits
Cu	Herbage	**6**–8	**6**–8	Diet Mo < 1.5 mg/kg DM
	Forage	**4**–6	**4**–6	

CRD: copper responsive disorder; Cu: copper; Mo: molybdenum; Fe: iron; S: sulfur; DM: dry matter.
Marginal bands indicate a probability of CRD that increases with proximity to the bold value.

Two studies with cattle showed an interaction between copper and manganese (Legleiter and Spears, 2007; Hansen et al., 2009). It was suggested that excess manganese adds to the effect of excess mucosal iron by competitive binding at the DMT1 – one of the two transporters that carry copper across the gut mucosa – thereby inhibiting the absorption of copper (Hansen and Spears, 2008). Alternatively, it is possible that soluble manganese, like iron, can trap S_2^- in the rumen and impair absorption of copper at distal sites. While cattle are not normally exposed to such high levels of manganese as salts, they are exposed to manganese in soils.

Copper–zinc interactions, as described in the sections for monogastric animals, may also occur in ruminants. Under practical conditions, zinc is not a major factor affecting absorption of copper unless the diet contains at least 20-fold more zinc than what is recommended (NRC, 2001).

3.4.3. Incompatibilities of copper in feed

Fats and oils are important and relatively expensive components of animal feeds. Hydrolytic and oxidative rancidity of feed lipid components (intrinsically present or added) is major cause of reduction of feed quality, affecting nutritive value, taste, aroma, colour and texture, which in turn will influence animal performance and health (Shurson et al., 2015). Factors that accelerate these processes include elevated temperatures, the presence of cationic metals such as copper, the presence of oxygen and the degree of unsaturation of the fat; Cu(II) is an effective catalyst of free radical formation leading to oxidation of oils and fats (for a review on the different steps in lipid oxidation, see Kerr et al., 2015).

Several endogenous enzymes (lipase, lipoxidase, lipoxygenase) present in many plant seeds and by-products are important in breakdown, oxidation and rancidity of plant lipids. Hydrolytic lipase activity is very important due to its role in the initiation of oxidative rancidity as a free fatty acid is more susceptible to oxidation as compared to when present in a triacylglycerol configuration. Lipolysis and subsequent rancidity is usually not a problem in intact grain, stored at normal temperature and moisture levels. Dierick and Decuypere (2002) studied endogenous lipolysis in feed materials and complete feeds, stored at different conditions for several months. The results showed a release of free FA amounting to 50% of the total FA present in the triacylglycerols in milled feedstuffs and in compound feeds, even after a few weeks of storage at room temperature. Native protective compounds (e.g. tocopherols, lignans) may be lost during processing of feed materials. Specific antioxidants (butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), TBHQ, propyl gallates, ethoxyquin, mixed tocopherols) or chelators/sequesters (citric acid, phosphoric acid, copper in chelated forms or covalent bounds) may be incorporated in complete feeds in order to lower potential lipid oxidation. Deleterious effects on gut mucosal structure, immune status, performances and/or meat quality when feeding animals with mixed feeds, containing oxidised fat, are frequently described in the literature (Dibner et al., 1996; Engberg et al., 1996; DeRouchey et al., 2004; Liu et al., 2014b; Rosero et al., 2015; Shurson et al., 2015).

There is plenty of literature on Cu-induced/accelerated oxidation processes in food matrices and meat products. However, although copper is known as a very effective catalyst promoting free radicals leading to oxidation of fats and oils also in feeds, only a few studies have specifically addressed the oxidation potential of copper sources (from e.g. organic (chelated) vs inorganic, particle size, solubility in water) and levels, added as trace element to complete feed, on the lipid fraction during feed storage.

Miles et al. (1998) studied the effect of adding different copper sources (copper sulphate (coarse and fine), TBCC) and levels (25–300 mg Cu/kg feed) to complete feed, stored at 37°C for up to 20 days, on lipid oxidation, by measuring the PV (marker for first oxidation products, e.g. hydroperoxides) and anisidine values (AV) (marker for secondary oxidation products) in the feed. The AV and PV values of the diets were strongly and significantly affected by time, copper source and levels; even at the lowest copper inclusion level, these values increased significantly. At the highest inclusion level, the PV and AV values at day 4 of storage amounted to 27.4, 270.0, 27.9 meq/kg and to 124,

684, 116 AV values for the coarse, fine copper sulphate and TBCC, respectively. Coarse copper sulphate and TBCC (not water soluble) promoted oxidation much less than the fine copper sulphate. However, further work is needed to clearly clarify the effects of copper, present up to the maximum permitted level in complete feed, on lipid oxidation during storage at normal temperatures.

Stability of vitamins (A, D3, E, riboflavin) has also been studied in the presence of copper, added in different forms and concentrations to premixes/complete feeds (Hooge et al., 2000; Luo et al., 2005; Lu et al., 2010; Shurson et al., 2011). For the majority of results, copper supplementation reduces the stability of vitamins. However, the data are not informative/realistic for practical feeding as the supplementation levels applied (125–300 mg Cu/kg feed) were well above the maximum authorised copper levels in feeds.

3.5. Copper intake and effect on gut microbiota profile

EFSA launched a procurement procedure to outsource the data collection, based on an Extensive Literature Search (ELS), on the potential influence of copper intake on gut microbiota profile. The terms of reference provided comprised the review of the matter with particular consideration to piglets; besides pigs/piglets, two other species were initially considered (chickens and cows). The full ELS report (Jensen, 2016) was published. The results are briefly summarised below.

For piglets and pigs, 229 references were found, 28 were considered appropriate to be included in the ELS. In total, 34 different characteristics related to the gastrointestinal microbiota were investigated in the selected studies. The total copper concentrations after supplementation with different copper sources (inorganic and organic copper, copper-bound clay and copper nanoparticles) varied from about 15 to 375 mg/kg feed. The overall conclusion is that copper may affect the microbiota in the gastrointestinal tract (both in foregut and hindgut).

Few results were found on the effect of copper on the pig small intestinal microbiota (load, profile, metabolism). It should be stressed that performance in piglets/pigs is directly related to the host and microbial digestion in the small intestine (Vervaeke et al., 1979; Dierick et al., 1988). The hindgut normally does not essentially contribute to the final energy and nitrogen balance of piglets/pigs, except when fibre-rich diets are used (e.g. boars and sows). There is evidence from the literature and the present report that copper in feed at concentration above requirements (5–15 to 175 mg/kg feed) shares the same mode of action as conventional growth-promoting antibiotics (e.g. Kellog et al., 1964; Vervaeke et al., 1976; Dierick et al., 1986; Jensen, 1998, 2016): in general, a reduction of lactobacilli/lactic acid bacteria in the foregut, which are known to compete with the host for nutrients (glucose, amino acids) and a reduction of clostridia and coliforms/*Escherichia coli*, in the foregut, which are more related to gut pathogenesis or less wanted microbial enzyme activities (e.g. β-glucosidase, β-glucuronidase). Also other parameters related to the load, profile and metabolic activity of the foregut microbiota (e.g. gut histo-morphology: villus height/crypt depth ratio, gut and plasma diamino oxidase activity, plasma D-lactic acid concentration) may be positively influenced by supplemental copper.

In the hindgut/faeces, the population of clostridia and coliform bacteria in most studies seems to be significantly (> 1 log CFU/g) reduced by dietary copper at concentrations below 50 mg/kg. At higher concentrations (> 170 mg/kg feed), a comparable effect was found on clostridia (only one study) but not in most studies on coliforms. It appeared that at these concentrations the population of lactobacilli in piglets as well as in growing pigs was significantly (> 1 log CFU/g) reduced in half of the studies. One study (49 mg Cu/kg) in pigs for fattening showed a significant (> 1 log CFU/g) reduced number of ureolytic bacteria in the colon after 3, 9 and 12 weeks.

For chickens, 106 references were found, 17 were considered appropriate to be included in the ELS. In total, 20 different characteristics related to the gastrointestinal microbiota were investigated in the selected studies. The copper concentrations from different copper sources (inorganic and organic copper, copper-bound clay and copper nanoparticles) varied from 8 to 375 mg/kg feed; however, only three studies were made with copper concentrations in feed complying with EU legislation. The overall conclusion from the studies with broilers is that copper affects significantly (> 1 log CFU/g) the microbiota in the gastrointestinal tract. The population of clostridia in the small intestine of chickens for fattening and laying hens seems to be significantly (> 1 log CFU/g) reduced in most studies by concentrations of copper between 25 and 100 mg/kg diet. No consistent effect could be found on the population of lactobacilli and coliform bacteria in the hindgut.

For bovines, 114 references were found. None of them was considered appropriate.

The FEEDAP Panel concludes from the studies with pigs and broilers that copper present in feed above requirements may affect significantly the microbiota in the gastrointestinal tract (foregut and hindgut/faeces), comparable to the mode of action of conventional antimicrobial growth promoters. For bovines no conclusions can be drawn.

3.6. Influence of copper in animal nutrition on the development of antibiotic resistance in bacteria

In the recent scientific literature, attention has been paid to the prevalence of antibiotic and copper resistance genes and especially to the assumed linkage between them and their regulation, in bacterial communities potentially exposed to high concentrations of copper. Two aspects should be considered: (a) the copper supplementation to the animals and its influence in the gut microbiota and (b) the copper in soil and development of antimicrobial resistance of soil bacteria. The first results were published in 2012 as part of an opinion of the FEEDAP Panel on the safety and efficacy of cupric sulphate pentahydrate for all animal species (EFSA FEEDAP Panel, 2012): a SLR on and a literature search on 'Copper in soil and development of antimicrobial resistance of soil bacteria'.

For the current opinion it appeared necessary to update the previous literature reviews.

3.6.1. Influence of copper on antibiotic resistance of gut microbiota in pigs (including piglets)

EFSA commissioned the University of Gent (Belgium) to assist in the update of the SLR conducted in 2012 on the 'Influence of Copper on antibiotic resistance of gut microbiota on pigs, including piglets'. Also, the literature review on the influence of copper on the development of antimicrobial resistance of bacteria in the environment was updated accordingly. The full report was published (Van Noten et al., 2016). A total of 901 references were examined to assess the influence of copper supplemented diets on copper and antibiotic resistance of gut microbiota in pigs (including piglets). Merely 33 references were found eligible to answer this review question. The conclusions are given below.

> 'The total number of studies managed during the first SLR was very low (only 22 studies). The current update of this review could add 11 more. This resulted in 11 field studies, 10 environmentally controlled studies and 12 cross-sectional studies. Furthermore, the methodological quality of the studies for the purpose of the present review was in general inadequate. From the 11 field studies, only three showed a good methodological quality. If all field studies are considered, the relation between copper supplementation and copper resistance appears equivocal. However, when only the results of the three studies with good methodological quality are evaluated, it can be concluded that an increased supplementation of copper (125 ppm compared to 16.5 ppm and 208 ppm compared to 6.4 ppm) will result in an increased resistance to copper sulphate of the enterococci population of piglets. In general *E. faecium* strains are more likely to acquire copper resistance than *E. faecalis* strains. In addition, the presence of the tcrB gene seems to correlate well with copper resistance. The transferability of the tcrB gene to enterococci from the same or other species was demonstrated.
>
> Resistance to erythromycin is most probably associated with this acquired copper resistance. The coselection of copper and erythromycin became evident from the environmentally controlled studies as well. Both resistance genes are present in close proximity on the same plasmid, which makes cotransfer of both genes plausible. This close genetic proximity is also the case for vancomycin and copper resistance, although the field studies did not result in conclusive evidence that copper resistance is associated with vancomycin resistance.
>
> These conclusions only concern gram positive bacteria. Reliable data regarding gram negative bacteria and especially *E. coli* are very scarce. According to our general impression, feeding elevated copper levels and the presence of the pcoD/A gene are presumably not related to higher copper resistance.
>
> All the cross-sectional studies showed rather poor methodological quality. This was mainly due to the selection of isolates that was biased or not representative for this review question or because of the limited information on the copper levels to which animals were exposed. Therefore, it is very difficult to draw conclusions from these studies.

To conclude, the limited number of studies available from the SLR, and the limitations in terms of results and methodological quality do not unequivocally allow to demonstrate the absence of a correlation between copper supplementation above requirements and development of antibiotic resistance in pigs under commercial farm practices. In addition, there is undoubtedly a genotypic relationship between both. More field studies are needed to address the issue, especially for the gram negative bacteria. For the time being, and in view of the inconclusive results, the recommendation might be not to increase the levels of copper supplemented in feed above allowances. Furthermore, considering the increasing research and interest on the matter in the latest years, it is recommended to repeat the SLR in future considering new approaches, if necessary'.

Based on the above, the FEEDAP Panel concluded that a co-selection in the gut bacteria for resistance to copper and resistance to erythromycin, likely at copper supplementation doses between 125 and 250 mg/kg feed, cannot be excluded; it has been demonstrated for Gram-positive bacteria (namely enterococci) but not for Gram-negative bacteria. The relevance of this finding is still uncertain.

3.6.2. Influence of copper on the development of antimicrobial resistance of bacteria in the environment

A literature search conducted by searching in three databases (AGRIS, ISI Web of Knowledge and PubMed) identified a total of 556 records.[20] Specifications of the literature search can be found in Appendix E. After screening title and abstract and considering only papers published after 2010, and dealing simultaneously with copper and soil/environment, 41 references were considered appropriate. The publication of Berg et al. (2010), provided by the EC, was given particular consideration.

Large reservoirs of antimicrobial resistance genes exist in bacterial communities from both natural and man-managed environments (Wright, 2007), and work suggests that these environmental reservoirs are important sources of new resistances in clinical settings (Smith et al., 2005; Martinez, 2009). The terrestrial reservoir of antimicrobial resistance (i.e. the soil bacterial resistome) is bound to be of major significance due to its size, diversity and placement in the human food chain (Baker-Austin et al., 2006; Wright, 2007). Indeed, research indicates that the soil bacterial resistome has expanded significantly during the antibiotic era (Knapp et al., 2010). Anthropogenic activities may greatly accelerate expansion of the soil bacterial resistome. This may occur via soil deposition of antibiotic-resistant bacteria and antimicrobial resistance genes present in animal manures and other faecal waste products (Pruden et al., 2006; Binh et al., 2008; Heuer et al., 2008). Alternatively, antibiotic residues reaching the soil environment may select for antimicrobial resistance (Brandt et al., 2009).

Selection for antimicrobial resistance in soil and water microbiota (also sewage sludge) is also possible under a selection pressure generated by compounds other than antibiotics. Bioactive compounds such as detergents, biocides, organic solvents and metals have been suggested to select for bacterial strains showing an increased expression of multidrug resistance determinants and thereby co-select for antibiotic resistance (European Commission, 2009).

Heavy metals in the environment result mainly from industrial and agricultural/aquacultural sources, the latter as feed additives, organic and inorganic fertilisers, pesticides and anti-fouling products. As the retention of trace elements (heavy metals including copper) in food-producing animals is approximately below 5% of the total consumed, the majority of ingested trace elements is excreted and found in the manure. For pig operations, heavy metals are found in the liquid fraction of the anaerobically digested swine manure and therefore also found in wastewaters. A correlation has been noticed between heavy metal concentrations in soil and the rate of application of pig manure (see review of Georgescu, 2014). Studies in the environment of broiler feedlots showed correlations between copper and resistance to different antibiotics. He et al. (2014) concluded that the presence of copper in feeds or environmental medium could be one of the important driving factors in the selection of antibiotic-resistant genes in the feedlot environments. According to Burridge et al. (2010), fish farmers use copper-containing products to prevent fouling of farm cages and nets; some cages are reported to be made from copper alloys. The enrichment of aquaculture sediments with copper is described by Smith et al. (2005).

The discharge of heavy metals together with antibiotics from agriculture and animal production-linked ecosystems to the environment may cause a combined effect of selection and co-selection

[20] The references included also four papers submitted by Authorities from European countries following a call for data (see Section 2.1) and four references cited in Appendix B, section 3 of the FEEDAP Opinion on Safety and efficacy of cupric sulphate pentahydrate (see EFSA FEEDAP Panel, 2012).

towards antibiotic-resistant bacteria. Therefore, soil and water compartments impacted by agriculture and aquaculture are critical foci of the evolution of antibiotic-resistant bacteria and require special scientific consideration.

In samples of soil heavily contaminated with metals, 36 out of 46 heterotrophic bacterial isolates showed a multiple metal and antimicrobial resistance (Krishna et al., 2012). Similar results were obtained by Malik and Aleem (2011) on the resistance pattern of *Pseudomonas* spp. in river water and soil irrigated with wastewater or groundwater. The majority of *Pseudomonas* isolates exhibited resistance to multiple metals and antibiotics and resistance was transferable to a recipient *E. coli* strain by conjugation.

Simultaneous resistance to copper and antibiotics has been observed for several bacteria species from different environmental compartments (e.g. in soil: Abo-Amer et al. (2013), Joshi and Modi (2013), Abo-Amer et al. (2014), Jechalke et al. (2015), Sevim and Sevim (2015); in sewage sludge: Heck et al. (2015); in aquatic environment (river and lake): Osman et al. (2010), Martins et al. (2014); in industrial effluents: Kaur et al. (2015); in coastal water: Beleneva et al. (2011); in sediments: Kacar and Kocyigit (2013)). However, most of the available evidence is, at best, circumstantial because the vast majority of studies rely on correlations between metal and antibiotic resistance genes (ARG) in bacterial isolates and on environmental studies where the selective agent cannot be identified with certainty. Such a correlation was also found in bacterial isolates from Antarctic shallow sediments where pollution from both chemicals and drugs is scarce (Lo Giudice et al., 2013). In sediments and soil samples collected from two Antarctic islands (Tomova et al., 2015), multiple antibiotic resistance was detected in 67% of the sediment isolates and 92% of the soil isolates. Most strains were resistant to copper (and lead, nickel and zinc). Plasmids were detected in 21% of the isolates. In studies with archived Scottish soil samples (Knapp et al., 2011), a bivariate correlation analysis showed that eight of the eleven quantified ARG positively correlated with soil copper levels: five ARG being highly significant ($p < 0.05$), including tet(M), tet(W), blaOXA, erm(B) and erm(F) (all genes were normalised to 16S-rRNA gene abundances).

In contrast, there are also studies in which no correlation could be shown (e.g. in urban waste water: Gao et al. (2015); in surface water: De Niederhäusern et al. (2013); in tannery effluents and irrigated soil: Alam et al. (2011); in hospital and outdoor strains of *Pseudomonas aeruginosa*: Deredjian et al. (2011); in waste dumps of mines: Mathiyazhagan and Natarajan (2012); in industrial areas: Wani and Irene (2014)). It is possible that, in these cases, the pressure exerted by exposure to heavy metals was not strong enough to stimulate further survival strategies like antimicrobial resistance.

To try and establish which heavy metals are likely to co-select for antimicrobial resistance in soil and water, Seiler and Berendonk (2012) reviewed the available data on heavy metal pollution, heavy metal toxicity, heavy metal tolerance and co-selection mechanisms. The authors also assessed the risk of metal-driven co-selection of antimicrobial resistance in the environment based on heavy metal concentrations that potentially induce this co-selection process. Analyses of the data indicated that agricultural and aquacultural practices represent major sources of soil and water contamination with moderate to highly toxic metals such as mercury, cadmium, copper and zinc. If these metals reach the environment and accumulate to critical concentrations, they can trigger co-selection of antimicrobial resistance. The authors specified the minimum heavy metal concentration, which correlates with a detection of increased bacterial antibiotic resistance, as the minimum co-selective concentration (MCC) of a metal.

In the natural water environment (water and sediment), copper (and cadmium, nickel, mercury, cobalt, lead and zinc) frequently reached levels that exceed their respective MCC values and therefore, could drive co-selection. However, the results of the MCC analysis for such data need to be carefully interpreted, mainly because positive correlations between metal levels and antimicrobial resistance may be misleading. This would be the case, if another selection pressure (e.g. by antibiotics) would be the trigger of the observed selection. Seiler and Berendonk (2012) concluded that copper in concentrations toxic to bacteria reaches the environment and accumulates to selective concentrations. Additionally, it can trigger co-selection of antimicrobial resistance because responsible co-selection mechanisms that mediate resistance to these heavy metals – and clinically as well as veterinary relevant antibiotics – have already been described. The authors noted that more research is necessary in order to better assess the risk of co-selection of antibiotic and heavy metal resistance.

Berg et al. (2010) studied the impact of copper pollution as a selective driver for the spread of antibiotic resistance in soil. Bacteria were extracted from a heavily contaminated soil (for more than 80 years; 150-fold higher in copper than the control soil) with copper sulphate, and from a corresponding control soil. Pollution-induced bacterial community tolerance (PICT) to copper and a

panel of antibiotics was determined by a cultivation-independent approach based on [3H] bromodeoxyuridine (BrdU) incorporation into DNA and by resistance profiling of soil bacterial isolates on solid media. High copper exposure selected not only for Cu-tolerant bacterial communities but also co-selected for increased community-level tolerance to tetracycline and vancomycin. Cu-resistant isolates showed a significantly higher incidence of resistance to five out of seven tested antibiotics (tetracycline, olaquindox, nalidixic acid, chloramphenicol and ampicillin) than Cu-sensitive isolates. The data suggest that soil copper exposure co-selects for resistance to clinically important antibiotics (e.g. vancomycin) at the bacterial community level. Co-selection for antibiotic tolerance in copper-polluted soils was studied in a laboratory experiment (using leucine incorporation) by Fernandez-Calviño and Baath (2013). The PICT found in copper-polluted soils to the antibiotics tetracycline, tylosin and vancomycin was observed at higher copper pollution levels than PICT to copper.

The increasing number of publications on microbial resistance against antibiotics and other agrochemicals like trace elements in the terrestrial environment stresses the importance that is allocated by science to this development. A large and possibly increasing reservoir of antibiotic resistance in the environment presents a clear hazard as resistance can be transferred, e.g. by conjugation, to pathogenic microorganisms. Both resistance against copper and against antibiotics are found associated globally, not only in areas heavily polluted with metals but also in areas like Antarctic water without a pollution history. Consequently, it remains unclear whether heavy metals like copper are one of the drivers of developing antibiotic resistance. In fact, it seems that, in general, when bacteria encounter adverse conditions, such as high levels of potential toxicants, dormant survival mechanisms (tolerance to adverse environmental conditions) are triggered. There is no doubt that a high copper content in manure represents a challenge to bacterial survival and is one that continues because of the resulting higher copper concentrations in soil (and surface water), at least in the limited surroundings of piggeries. This may be followed by increased resistance to antibiotics in these areas. However, a sufficiently clear demonstration of this chain of events is currently not available. It is noted that, if no action is taken now and the potential progression including a risk of resistance gene transfer to human pathogens is demonstrated in the future, worthwhile years of counteractive measures will be lost.

3.7. Proposals for maximum copper contents in complete feed

The details of nutrient requirement data for the different target animals are usually taken into consideration when formulating a complete feed. Feed business operators base calculations for nutrients in diets on averages/medians – intended primarily not to fall below the animal's requirements – and consider the maximum contents set by legislation. Maximum contents in feed are set for trace elements for different reasons, which include safety for the consumer, the target species and the environment. The request of the EC covers the appropriateness of the CAMC when compared with the requirements.

The proposal for a potential modification of the CAMC of copper has to consider the different aspects discussed in this document. The NPMC shall be high enough to ensure health, welfare and performance of healthy target animals. The NPMC must therefore be above requirements considering age, genetics and physiological state (determined by animal performance, including growth, pregnancy, lactation, egg production, wool and fur production as well as physical work).

The principle of allowances in animal nutrition is equivalent to derivation of a Population Reference Intake (PRI)/Recommended Dietary Allowances (RDA) in humans. A PRI/RDA is the average daily dietary intake level that is sufficient to meet the nutrient requirement of nearly all (97.5%) healthy individuals at a particular life stage and gender group (EFSA NDA Panel, 2010). Therefore, this approach can also be used to derive the NPMC.

- If the distribution of requirements in the group is assumed to be normal, then the PRI/RDA can be calculated from the requirement and the standard deviation (SD) of requirements as follows: PRI/RDA = Requirement + 2 SD (EFSA NDA Panel, 2010; Health Canada).[21] However, it is not possible to calculate an inter-individual variation in copper requirement for all animal species and therefore variability has to be estimated.
- If data about variability in requirements are insufficient to calculate a SD, a coefficient of variation (CV) for the requirements of 10–20% can be assumed (EFSA NDA Panel, 2010). If 10% is taken to be the CV, then twice that amount when added to the requirements is

[21] Health Canada, Official webpage. Food and Nutrition. Dietary Reference Intakes. http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/index-eng.php

defined as equal to the PRI/RDA. The resulting equation for the PRI/RDA is then Allowance = 1.2 × requirement. This level of intake, statistically, covers the requirements of 97.5% of the population.

- It is concluded that copper allowance is calculated from 1.2 times the requirement.

Allowances also have to take into consideration variation in the availability of copper sources used for feed supplementation (see Section 3.4.1). Most copper-containing compounds show bioavailabilities in all animal species similar to copper sulphate. However, cupric oxide and cupric carbonate (and cuprous iodide in poultry) showed a lower and more variable relative bioavailability (RBV). It is concluded that copper allowance in animal feed needs to include an additional 30% of the requirement to account for differences in bioavailability of background copper, arriving at a final factor of 1.5 (1.2 + 0.3 ~ 1.5).

The RBV of background copper in diets for poultry can be estimated to be 50% and < 50% in pigs. However, NPMC should generally also consider the interactions of copper with other nutrients, minerals or additives, if these have not already been taken into account when establishing requirements. Because copper in feed materials interacts with other nutrients and minerals, particularly with molybdenum and sulfur in ruminants, it is difficult to precisely estimate the RBV of copper in the basal diet. Therefore, for the following calculations, background copper is not taken into account as an available source for meeting the requirements but providing a reserve for a potentially increase need because of the presence of antagonists.

The NPMC should be feasible under the practical conditions of the feed manufacturing industry. This is considered by rounding the mathematically derived NPMC to practical figures and thus, establishing three groups of NPMC.

The following aspects were considered when proposing a modification of the CAMC: (i) the NMPC should not be higher than what is inevitably necessary; (ii) as there is no substantial retention of copper in the animal body, the potential reduction in dietary copper will be reflected in a reduced environmental load; and (iii) although it is presently not scientifically fully demonstrated that high copper, as in case of piglet feed, triggers the development of bacterial resistance to antibiotics in the gut and/or the environment, the 'As Low As Reasonably Achievable' (ALARA) principle is supported.

The NPMCs are summarised in Table 10; only the animal categories listed in this table, for which requirement data are reported in Section 2.1, could be considered.

As no requirement could be identified for crustaceans, it is recommended to maintain the current maximum copper content of 50 mg/kg feed.

Table 10: Newly proposed maximum copper contents in complete feed for target animals, expressed as mg Cu/kg complete feed

Target species, animal category	R[(a)]	1.5 × R	Background	NPMC[(b)]	CAMC[(b)]
Chickens for fattening, reared for laying	8	12	10	25	25
Laying hens, breeder hens	8	12	10	25	25
Turkeys for fattening, 0–8 weeks of age	10	15	10	25	25
Turkeys for fattening, from 8 weeks of age onwards	6	9	10	25	25
Other poultry	8	12	10	25	25
Piglet, weaned	8	12	10	25	170
Pigs for fattening	6	9	10	25	25
Sows	10	15	10	25	25
Calves – milk replacer	10	15	5	15[(c)]	15
Cattle for fattening	8.8*	13.2	10	30[(d)]	35
Dairy cows	8.8–13.2*	13.2–19.8	10	30	35
Sheep	7*	10.6	10	15[(e)]	15
Goat	7–22*	10.6–33	10	35	25
Horses	8.8*	13.2	10	25	25
Rabbits	5	7.5	10	25	25

Target species, animal category	R[(a)]	1.5 × R	Background	NPMC[(b)]	CAMC[(b)]
Salmonids	5	7.5	10	25	25
Other fish	8	12	10	25	25
Crustaceans				50	50
Dogs	12	15.6[(f)]	10	25	25
Cats	10	13[(f)]	10	25	25

(a): R: requirement.
(b): NPMC: newly proposed total maximum contents of copper in complete feed.
CAMC: currently authorised maximum content of copper in complete feed.
(c): As copper in milk-based diets shows a high bioavailability and does not contain substantial amounts of copper antagonists, the background level (5 mg/kg) is not added to the allowance (15 mg/kg).
(d): Considering cattle feeding on pasture, a potentially high amount of copper antagonists in forage is taken into account.
(e): NPMC is limited by the MTL; dietary concentrations above 15 mg/kg could provoke CCP.
(f): No requirement data available, the allowance (12 and 10 mg/kg for dogs and cats, respectively) is therefore only multiplied by 1.3 to consider different bioavailabilities of copper supplements.
(*): Adjusted from dry matter to complete feed with 88% dry matter.

The proposed reduction in the CAMC would not have an impact on the basal composition (feed materials) of diets for the target animals. Considering a background content of 10 mg Cu/kg diet and the 90th percentile values of copper concentrations in feed for piglets (168 mg/kg), it is concluded that 158 mg results from copper supplementation; the proposed reduction of 145 mg (170 minus 25) is lower than the practised supplementation at the 90th percentile.

The proposal for a reduction in the maximum dietary copper in dairy cows is supported by a recent comprehensive report on trace element supply including copper by TMR in Saxonian dairy farms (Steinhöfel et al., 2013).

The Norwegian Food Safety Authority made some proposals for a reduction in the CAMC to the European Commission (see footnote 3). The Authority proposed the following maximum copper contents: for cattle 30 mg/kg; for pigs, poultry and horses 15 mg/kg; for piglets 35 mg/kg. The Authority further recommended maintaining the current CAMC for sheep and pre-ruminant bovines, (15 mg/kg) and for fish (25 mg/kg). These proposals are generally in line with the NPMC (see Table 10).

3.8. Consequences of the proposed maximum copper contents in feed

3.8.1. Consequences on health and welfare of target animals

As previously stated, the NPMC are based on the more recent available data on copper nutritional requirement and tolerance studies conducted in modern breeds, up-to-date feeding conditions and diet composition.

The FEEDAP Panel considers that for most monogastric species and categories (except piglets) the CAMC are still valid and no changes have been proposed. These CAMCs are well above the requirements and ensure animal health, welfare and productivity. Moreover, most of them are in the range of recommendations from industry for copper content in complete feed.

The only drastic reduction in the CAMC (170 mg/kg) to a NPMC (25 mg/kg) is proposed for weaned piglets up to an age of 12 weeks. Reviewing the literature (see Section 3.2.4), distinct adverse effects have to be expected when following that proposal in practice; the first consequence would be a reduction in growth performance, likely accompanied by reduced faecal consistency. However, growth promotion and improved faecal consistency are not the physiological effects of meeting the animals' requirements for copper; they are considered rather as pharmacological effects, similar to that of 2,000–3,000 mg Zn (from zinc oxide)/kg feed and of growth-promoting antibiotics for piglets. The FEEDAP Panel gives consideration as to whether such an extra-nutritional effect should be covered and authorised by veterinary medicinal product regulation and not by feed regulations. The Panel also notices that there is a variety of alternatives in compounding complete feed which would have the same positive effect on growth as the high copper. Furthermore, the copper reduction in piglets feeding could trigger an increase in the use of antibiotics. However, the Panel is convinced that knowledge and experience of feed compounding industry would allow feed formulations containing elements (feed additives, feed materials) which fully substitute the growth-promoting effect of copper; an additional compensation may be reached by improving hygienic and sanitary conditions. Norwegian

data may serve as an example: Norway has an exception from the EEA-agreement and has a national limit for maximum copper content in feed for piglets of less than 12 weeks of age at 35 mg/kg complete feed. A comparison with the economic data from 2012 of production of weaned piglets from five EU MS (Denmark, Germany, UK, Spain and Sweden) did not identify a disadvantage of the lower maximum dietary copper (ADG (g), MS-range: 291 (Spain)–489 (UK); Norway: 504. Feed/gain, MS-range: 1.6 (Sweden)–1.77 (UK); Norway: 1.63. Mortality (%), MS-range: 2.0 (Sweden)–3.4 (Spain); Norway: 1.7).[22] The comparison shows that high dietary copper in feeding piglets is not a unique condition for reaching optimum economic production results.

Several articles on alternatives for antimicrobial growth promoters, including copper, in pig feeding, are available in the literature (e.g. DIAS Report, 2004; Gallois et al., 2009; De Lange et al., 2010; Kil and Stein, 2010; Vondruskova et al., 2010; Heo et al., 2013; Thacker, 2013; Poulsen et al., 2015; Suiryanrayna and Ramana, 2015). These alternatives include feed additives (e.g. organic acids, medium-chain FA, pre- and probiotics, essential oils and other plant extracts), feed materials (e.g. butyric acid and oils containing medium-chain FA), feeding systems (e.g. fermented liquid feeding) and management systems (e.g. segregated early weaning). They all have an influence on the gut histomorphology (and function) and intestinal microbiota similar to that observed after the administration of high copper and antibiotics, which would help to explain the growth-promoting effect.

In the case of bovines, recent studies indicate that in some circumstances this animal species can be less tolerant to copper than traditionally thought and cases of subclinical chronic copper toxicity (by excessive hepatic copper accumulation) are frequent across EU (see Section 3.2.3). The NPMC is expected to better protect bovines of suffering from excessive hepatic copper accumulation. Higher levels of dietary copper would only be required to meet the copper physiological needs when high levels of copper antagonists (molybdenum and sulfur) are present in the diet, but this is not the case across the EU. The FEEDAP Panel considers that caprines might have higher copper requirements than traditionally thought and, consequently, proposes to increase the CACM (25 mg/kg) to 35 mg Cu/kg.

Finally, the FEEDAP Panel notes that interactions with other trace elements and certain dietary constituents deserve increased attention when the use levels of dietary copper are reduced, particularly in ruminants. However, feed business operators have full access to the relevant databases which are used to calculate feed formulations on the basis of the most updated information.

3.8.2. Consequences on consumer supply

Dietary Reference Values (DRVs) for humans have not been established for copper because of the limited quality and quantity of the available information; instead, Adequate Intakes (AI) have been set by the Nordic countries, Germany, France, the Netherlands, USA and Canada, and the SCF. An AI is 'estimated when a PRI cannot be established because an average requirement cannot be determined. An AI is the average observed daily level of intake by a population group (or groups) of apparently healthy people that is assumed to be adequate' (EFSA NDA Panel, 2015). The values set range between 1.0 and 3.5 mg/day for men and 0.9 and 3.5 mg/day for women. When the EFSA NDA Panel reviewed the DRVs for copper in 2015, again it was concluded that only AIs for copper could be set; there were still too few data on which to base DRVs. However, there was enough information to enable the NDA Panel to set different AIs according to gender. These AIs are shown in Table 11.

Table 11: Summary of Adequate Intakes for copper (EFSA NDA Panel, 2015)

Age/group	Adequate Intake values for copper (mg/day)	
	Males	Females
7–11 months	0.4	0.4
1 to < 3 years	0.7	0.7
3 to < 10 years	1.0	1.0
10 to < 18 years	1.3	1.1
≥ 18 years	1.6	1.3
Pregnancy		1.5
Lactation		1.5

[22] Data from Interpig 2012. Provided by the EC with the mandate.

The average copper intakes in the EU are summarised in Table 12.

Table 12: Ranges of average copper intakes in Member States of the EU (from EFSA NDA Panel, 2015)

Age/group	Range of average copper intakes (mg/day)
Infants	0.34–0.50
1 to < 3	0.57–0.94
3 to < 10 years	0.82–1.44
10 to < 18 years	0.98–1.92
≥ 18 years	1.15–2.07

The natural copper content of foods varies greatly. Representative food composition data are shown in Annex D (Table D.1). It can be seen that rich dietary sources of copper are liver, some seafoods (oysters), cocoa products, nuts (particularly cashew) and seeds.

The main food group contributing to copper intake is grains and grain-based products, except for infants for whom the main contributor to copper intake is food products targeted for the young population. Although grains and grain-based products do not have concentrations of copper as high as those reported for other food groups – such as offal, nuts, or liver – the high consumption of foods in this group (e.g. bread) and the large variety of products in which grains are included makes them the most important contributor to copper intake 19–40% in women and 26–44% in men.

The food group starchy roots or tubers and products thereof provide 5–10%, vegetables about 11%, fish and seafood up to 16% and eggs and egg products 1–11% of the dietary copper intake of European populations (EFSA NDA Panel, 2015). The contribution of water and water-based beverages to copper intake in various age groups was up to 12%. The copper content of drinking and potable water is influenced by natural mineral content, pH and a copper or non-copper supply system (NRC Committee on Copper in Drinking Water, 2000). Soft acidic water, especially when transported by copper pipes, has a higher copper concentration. It has been estimated that foods may account for 90% or more of copper intake in adults when the copper content in drinking water is low (< 0.1 mg/L). If the copper content is higher (> 1–2 mg/L), water may account for up to 50% of total intake. In infants, contribution of water to daily copper intake may be higher because they consume proportionally more water than adults (de Romaña et al., 2011).

The food group meat and meat products is an important contributor to copper intake, with an average contribution up to 19% in males and up to 16% in females. However, food consumption data from Finland, France, Germany, Ireland, Italy, the Netherlands, Sweden, and the UK indicate that liver and liver-based products contribute < 1% to daily consumption of copper in most age groups. The exceptions are the elderly and very elderly people in whom a percentage of contribution up to about 6% and 12%, respectively, has been reported (France and the UK) (EFSA NDA Panel, 2015).[23] It is also noteworthy that many consumers do not eat liver. Data extracted from the Comprehensive European Food Consumption Database (data from 19 MS countries) show that, on average, only about 3.3% of the European population consumes liver, with values up to 17% (Hungary). However, higher percentages of the population consume some liver-based products (e.g. 90% of the Danish population). These population patterns are contained within the overall assessment of liver consumption and copper exposure discussed earlier in this section.

The issue relating to consumer safety in this opinion is that a reduction in copper added to animal feed might reduce the copper intake of consumers. At the levels of exposure arising from any reduction of the copper contents of feed provided for animals, the copper content of their muscle and liver would not be altered and would have little impact on consumers' diets. Additionally, if the copper content of liver were reduced as a result of a change in feeding practice, liver is such a small source of dietary copper, even in those consumers who eat liver and liver products, that any alteration in the copper content of liver as food would also have a negligible impact on consumer intakes.

The FEEDAP Panel concludes that the reduction of the copper CAMC for some categories of food-producing animals to the NPMC values would not be likely to have any consequences on the consumers' intake of copper and is of no concern for the safety of the consumer.

[23] Data extracted from the EFSA Food Consumption Database: https://www.efsa.europa.eu/en/food-consumption/comprehensive-database

3.8.3. Consequences on the environmental load

Most of the copper consumed by farm animals appears in faeces, particularly when pharmacological 'growth-promoting' levels are applied (see Section 3.2.4). This can result in substantial excretion of copper that is added to the agricultural soil via manure application. Particularly, in livestock-dense areas and regions, this can result in annual accumulation of copper in soil that gives rise to long-term environmental concern (Monteiro et al., 2010).

The release of copper from different anthropogenic sources in Europe was analysed in the EU voluntary Risk Assessment Report (RAR) of copper (ECHA, 2008). The FEEDAP Panel could not identify any more recent reports on the topic. The total amount of copper used in the EU in 1999 was 4.3 M tonnes (ECHA, 2008) and 56% of this went into electrical cables and electrical equipment. The use of copper chemicals (as sulphate, oxide, chloride) in 1999 was 32,400 tonnes (ECI, 2003, cited in ECHA, 2008), which corresponds to 0.7% of the total copper use. Out of this amount, 19% (6,200 tonnes/year) was annotated as 'cattle feeds', although it is unclear if this figure rather refers to all 'animal feeds' because no other categories were included in the budget (ECHA, 2008). Thus, the amount of copper used for feed supplements is very small in comparison with the total use of copper, but it is an important source of copper in agricultural soils. However, it should be noted that also other uses of copper, e.g. in fertilisers or as pesticides, contribute to copper load of soil.

EFSA commissioned a study on the impact of zinc and copper in farm animal manure on the EU environment (Monteiro et al., 2010). In some soil types, potential risk was identified for soil organisms, particularly following long-term (50 years) application of piglet manure. However, it was noted that only two out of the seven soil types considered would be expected to coincide with piglet farms (Monteiro et al., 2010); the use of copper in fish feeds was considered not to give rise to concern.

An estimate was made of the reduction in copper input to the environment following the potential implementation of the NPMC for piglets, cattle for fattening and dairy cows. The NPMC refers to complete feed and, therefore, calculations for complementary feeds were not possible. The amount of copper going into animal feed production was calculated by using compound feed production data (FEFAC, 2016). A comparison was made between feeds containing copper concentrations at the CAMC and the NPMC. The total EU production volumes of feed for individual animal categories (e.g. piglets) were corrected for lack of detail in reporting from some countries (six to eight MS out of 25 reporting compound feed production) by assuming an equal mean for the MS reporting feed production for a certain category and for all 25 MS generally reporting. To assess the reduction in environmental copper contamination, it was further assumed that the copper content in animal tissues and products will not be substantially affected by changing copper in feed to the NPMC. Consequently, in this very simplistic model, the absolute reduction in the copper eaten by animals is equal to the reduction in environmental copper emission via manure. It was estimated that the amount of copper entering the EU environment per year via farm animal manure would be reduced by up to 1,000 tonnes following introduction of the NPMC (see Appendix F). Reducing copper in feed for piglets would have the greatest impact by far, resulting in an estimated 860 tonnes less copper being spread on fields.

Another estimate for piglets only, based on the production of 250 million pigs in Europe/year (European Commission, 2016) and an excretion of 14.3 g Cu/pig, gives a saving in the environment of 6.5 g/pig (Jondreville et al., 2002), thus resulting in 1,600 tons less copper per year.

From an average of the two above calculations ((860 + 1,600)/2 ≈ 1,200 tonnes), it can be assumed that only the reduction in the maximum copper content in piglet feed from 170 to 25 mg/kg could result in about 20% savings of total copper emissions from farm animal production. Thus, locally where manure from piglets is spread on land, the reduction in the CAMC to the NPMC would undoubtedly have a significant impact on the concentrations of copper in the environment of piggeries. However, given that the total amount of copper used in animal feeds is very small compared to overall use and emission, implementation of NPMC will likely do little to reduce the copper levels in the wider environment.

3.9. Maximum residue limits of copper in food of animal origin

As several compounds of copper (copper hydroxide, copper oxychloride, Bordeaux mixture, tribasic copper sulphate and copper oxide) are also used as inorganic pesticides (fungicide/bactericide), MRLs have been established under Regulation (EC) No 396/2005[13] (Annex IIIA, temporary MRLs). From a comparison of these MRLs and the tissue concentrations reported in the literature after the use of copper-supplemented feed diets made by the FEEDAP Panel, it became evident that MRLs considerably

underestimate the occurrence of copper in liver from ruminants (and water fowl), and simultaneously reflect unrealistically high concentrations in all other tissues and products (EFSA FEEDAP Panel, 2012). Several data on the copper concentration in food of animal origin, tissues and products, are listed in Annex D. They confirm that the MRLs may be exceeded, particularly in the case of ruminant liver.

A report on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products during 2014 (EFSA, 2016) confirmed the above appraisal.[24] Samples analysed for copper were only available from two MS (Czech Republic and Germany). About 45% and 60%, respectively, of the analysed bovine liver samples exceeded the MRL; however, only about 3% and 11% did in pig liver samples, respectively (for details, see Annex E).

In the framework of the pesticide legislation, the MRLs for copper compounds are currently under review by EFSA, based on a draft report prepared by the Rapporteur MS France.

The FEEDAP Panel wants to give its general position on the usefulness of the MRLs for essential micronutrients. Trace elements such as copper fulfil vital functions in living organisms. As they are required as constituents of the feed, they would consequently be also found in tissues and products of animal origin. The EU feed legislation has also established maximum contents for these trace elements in order to preserve the safety of target animals, consumer, users and/or the environment. It is the general position of the FEEDAP Panel that for essential trace elements no MRLs should be set; they are simply not necessary as other measures to limit consumer exposure can be taken, for example a restriction of their concentration in animal feed.

As many compounds of trace elements are also considered as pharmacologically active substances, they have been assessed by the European Medicines Agency (EMA) by its Committee for Medicinal Products for Veterinary Use (CVMP), also under the particular aspect of whether MRLs would be necessary or not; no MRLs have been recommended by CVMP (where necessary, the Committee considers alternative measures to protect consumer safety, e.g. setting withdrawal periods). Consequently, Regulation (EU) No 37/2010 classifies all (compounds of) trace elements as 'No MRL required'. In the context of copper compounds, EMA has delivered a report on copper chloride, copper gluconate, copper heptanoate, copper oxide, copper methionate, copper sulphate and dicopper oxide (EMEA, 1998) and another one on copper carbonate (EMA, 2016), which were regarded in Regulation 37/2010 and, more recently, in Regulation 2016/710, respectively.

The concentration of copper in the animal organism is efficiently regulated by homeostatic mechanisms, which include gastrointestinal absorption, the uptake and metabolism by the liver and biliary excretion into faeces. Copper toxicity ensues when the capacity of the homeostatic mechanisms is exceeded. Among edible tissues of animal origin, the highest concentration of copper is found in the liver (the main deposition organ), followed by the kidney and the muscle. Among products of animal origin, milk shows the lowest values (EFSA FEEDAP Panel, 2016).

The FEEDAP Panel is of the opinion that MRLs for copper should not be set for tissues and products of animal origin as the use of copper is safely regulated by feed legislation. The authorised maximum copper contents in complete feed would effectively prevent any increase of copper in feed and, consequently, in food of animal origin.

4. Conclusions

The FEEDAP Panel reviewed (i) the copper requirements of food-producing and pet animals, (ii) the copper concentration in feed materials and complete feed, (iii) the copper bioavailability, and (iv) the calculated background copper concentration of complete feed. Also considered were (i) the influence of dietary copper on gut microbiota profile and on the bacterial antibiotic resistance in target animals and (ii) the environmental occurrence of bacterial heavy metal tolerance (copper resistance) and resistance to certain antibiotics. The data collected supported the possibility of a reduction in some of the currently authorised maximum contents (CAMC) for total copper in feed.

The Panel developed an algorithm to derive NPMC from the requirement and the native dietary copper content. The NPMC (mg Cu/kg complete feed) comprised of maintained (m), decreased (d) and increased (i) values, and were: 15 for bovine before the start of rumination (m), 30 for other bovine (d), 35 for caprine (i), 15 for ovine (m), 50 for crustacean (m) and 25 for other animal species ((d) for piglets up to 12 weeks, (m) for all other species).

[24] Further details on non-compliant samples were retrieved from the Report on the implementation of National Residue Monitoring Plans in the Member States in 2014 (Council Directive 96/23/EC). Available online: http://ec.europa.eu/food/safety/docs/cs_vet-med-residues_workdoc_2014_en.pdf

The NMPC support health, welfare and economic productivity of target animals, except piglets; in weaned piglets, performance may be reduced.

The reduction in the CAMC for food-producing animals to the NPMC values would not be likely to have any consequences on the consumers' intake of copper and is of no concern for the safety of the consumer.

The reduction of copper in feed for piglets from 170 mg/kg to 25 mg/kg would have the capacity to reduce total copper emissions from farm animal production by about 20%. Thus locally, where manure from piglets is spread on land, the reduction in the CAMC to the NPMC would have a significant impact on the concentrations of copper in the environment of piggeries.

5. Recommendations

The two warning statements contained in *Other Provisions* for Copper in Regulation 1334/2003 should be maintained. No NPMC is proposed for sheep and the feed concentration for ruminants which is given under *Other Provisions* is a low copper level compared to the NPMC.

The FEEDAP Panel recommends enlarging the basis of copper monitoring in the liver of slaughtered bovines. Tracing back the results to the localised origin of the samples would help to better understand the conditions under which average values are exceeded.

The FEEDAP Panel further recommends implementing a monitoring of copper pollution from agriculture in areas in which food-producing animals are fed, with particular attention to the potential development of microbial antibiotic resistance in the environment. The data would help to identify any area under risk.

6. Remark

The FEEDAP Panel is of the opinion that MRLs for copper should not be set for tissues and products of animal origin, as also expressed by the EMA-CVMP. The use of copper is safely regulated by feed legislation. The authorised maximum copper contents in complete feed would effectively prevent any increase of copper in feed and, consequently, in food of animal origin.

Documentation provided to EFSA

1) Scientific Publication of Berg et al. (2010). (See References). Submitted by the European Commission. May 2015.
2) Document from the Norwegian Food Safety Authority on 'Copper supplementation of animal feed', including the papers of 'Copper and iodine in feed to fish' from NIFES, of 'Zinc, copper and iodine in feed for terrestrial livestock animals' from the Norwegian Veterinary Institute and the 'Interpig 2012'. Evaluation of change in maximum levels. Submitted by the European Commission. June 2015.
3) Data from European Countries concerning 'Allowance/Requirements levels of copper for animal species, defined by national scientific bodies' and 'Analyses of compound feed for all animal species/categories obtained during national official controls' received as reply to the ad-hoc questionnaires submitted throughout the Focal Points of the EFSA's Advisory Forum and the Chemical Occurrence Network platform. January 2016.
4) Data from Stakeholders concerning 'Industry recommendation of zinc supplementation and copper use level in all animal species categories in the EU' and 'Typical composition of complete/complementary feed for all animal species/categories' received as reply to the ad-hoc questionnaires submitted to the stakeholders via the EFSA's stakeholder platform. January 2016.
5) External Scientific Report. Assistance in the Update of the Systematic Literature Review (SLR): 'Influence of Copper on Antibiotic Resistance of Gut Microbiota on Pigs (including Piglets) '. Submitted by Van Noten et al. (See References). February 2016.
6) External Scientific Report. Extensive Literature Search on the 'Effects of Copper intake levels in the gut microbiota profile of target animals, in particular piglets'. Submitted by Jensen (See References). April 2016.
7) EFSA Internal Report. Technical assistance in retrieving and collecting data on non compliances on Copper from the EU residue database. June 2016.
8) EFSA Internal Report. Technical assistance in retrieving and collecting data on intake and food composition. June 2016.

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Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
ADME	absorption, distribution, metabolism and excretion
AFZ	Association Française de Zootechnie (French Association for Animal Production)
AI	adequate intakes
ALARA	As Low As Reasonably Achievable
ALT	alanine transaminase
ARG	antibiotic resistance gene
AST	aspartate aminotransferase
Atox1	Antioxidant 1 Copper Chaperone
ATP	adenosine triphosphate

ATTD	apparent total tract digestibility
AV	anisidine values
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BrdU	[3H]bromodeoxyuridine
bw	body weight
BWG	body weight gain
CAAS	Czech Academy of Agricultural Sciences
CAMC	currently authorised total maximum contents of copper in complete feed
CIRAD	French Agricultural Research Center for International Development
CCP	chronic copper poisoning
CNS	central nervous system
CP	crude protein
CTR	copper transporter
CRD	copper responsive disorders
Cu	copper
CVB	Centraal Veevoederbureau
CV	coefficient of variation
CVMP	Committee for Medicinal Products for Veterinary Use
Dcytb	duodenal cytochrome B
DMT	divalent metal transporter
DRV	dietary reference value
DW	dry weight
EC	European Commission
ECHA	European Chemicals Agency
ECI	European Copper Institute
EEA	European Economic Area
EFTA	European Free Trade Association
ELS	Extensive Literature Search
EMA	European Medicines Agency
EMEA	European Agency for the Evaluation of Medicinal Products
FA	fatty acids
FAO	Food and Agricultural Organization of the United Nations
FCR	feed conversion ratio
Fe	iron
FEDIAF	European Pet Food Industry Federation
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FEFAC	European Feed Manufacturers' Federation
FEFANA	EU Association of Specialty Feed Ingredients and their Mixtures
GfE	Gesellschaft für Ernährungsphysiologie
GLDH	glutamate dehydrogenase
GMT	gamma-glutamyltransferase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulfide
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
IFZZ	Instytut Fizjologii I Zywienia Zwierzat
INRA	Institut National de la Recherche Agronomique
Lys	lysine
MCC	minimum co-selective concentration
ME	metabolisable energy
Mn	manganese
Mo	molybdenum
MRL	maximum residue limit
mRNA	messenger ribonucleic acid
MS	Member States
MT	metallothionein

MTL	maximum tolerable level
MTT	Maa- ja elintarviketalouden tutkimuskeskus
MUFA	monounsaturated fatty acids
NDA	EFSA Scientific Panel on Dietetic Products, Nutrition and Allergies
NP	neuropeptide
NPY	neuropeptide Y
NPMC	newly proposed maximum content
NRC	National Research Council
PICT	pollution-induced bacterial community tolerance
PRI	Population Reference Intake
PUFA	polyunsaturated fatty acids
PV	peroxide value
RAR	Risk Assessment Report
RBV	relative bioavailability
RDA	Recommended Dietary Allowances
S	sulfur
SCAN	Scientific Committee on Animal Nutrition
SD	standard deviation
SGR	specific growth rate
SLR	Systematic Literature Review
STEAP2	six-transmembrane epithelial antigen of the prostate 2
TBARS	thiobarbituric acid-reactive substances
TBCC	tribasic copper chloride
TGN	trans Golgi network
TMR	total mixed ration
TBHQ	tertiary butylhydroquinone
UFA	unsaturated fatty acids
VSP	Videncenter for Svineproduktion
WGR	weight gain rate
Zn	zinc
Zn-SOD	zinc-superoxide dismutase

Appendix A – Description of studies on fish copper requirements

Mohseni et al. (2014a) exposed juvenile beluga (*Huso huso*) to diets containing 1, 3.5, 7.1, 9.7, 13, 25, 50 or 195 mg Cu/kg wet weight for 12 weeks. Hepatic Cu-Zn SOD activity was significantly higher in fish fed 9.7 and 131 mg Cu/kg diets compared to the other treatments. Levels of hepatic TBARS were significantly lower in fish fed 9.7 and 13 mg Cu/kg diets than in fish fed the other diets. The authors reported that the optimum dietary copper levels for beluga, based on weight gain, was between 10 and 13 mg/kg feed.

Wang et al. (2015) fed tongue sole (*Cynoglossus semilaevis*) diets containing 6, 8, 12, 24 or 56 mg Cu/kg for 8 weeks. Weight gain ratio, SGR,[25] feed:gain ratio (F/G) and protein efficiency ratio were significantly higher in fish fed the diet which contained 12 mg Cu/kg compared to fish fed the basal diet which contained 6 mg Cu/kg. Protease, amylase, lipase, copper-zinc superoxide dismutase (Cu-Zn SOD), and lysozyme activity initially increased and subsequently decreased with increasing dietary copper concentrations, and maximum activity of all parameters was observed in fish fed the 12 mg/kg diet. The copper requirement of tongue sole was estimated to be about 11–12 mg/kg diet based on growth and enzyme activities.

Purified diets with eight levels (2.1, 3.0, 4.2, 5.5, 6.1, 10.1, 14.0 or 19.6 mg Cu/kg diet) of copper were fed to juvenile hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) for 8 weeks (Shiau and Ning, 2003). Weight gain was highest in the group fed the 4.2 mg Cu/kg diet, followed by fish fed the 3.0 mg Cu/kg diet, and was lowest in fish fed the 19.60 mg Cu/kg diet. Blood haemoglobin (Hb) and haematocrit (Hct) concentrations were lowest in fish fed the two highest copper diets (14.0 and 19.6 mg/kg), followed by the unsupplemented control diet and highest in fish in the 4.2 mg Cu/kg group (Hb) and 4.2 and 5.5 mg Cu/kg diet groups (Hct). Plasma ceruloplasmin activity was highest in the 3.0 and 4.2 mg Cu/kg diet groups, followed by fish fed 5.5–14.0 mg Cu/kg diet and the control group, and lowest in fish fed the 19.6 mg Cu/kg diet. The dietary copper requirement in growing tilapia was found to be about 4 mg Cu/kg diet based on weight gain.

Lin et al. (2008) fed juvenile grouper, *Epinephelus malabaricus*, diets with 0.11, 1.7, 2.4, 4.4, 6.6, 9.0, 11 or 20 mg Cu/kg diet for 8 weeks. Weight gain and feed efficiency (FE) were higher in fish fed 4.4 and 6.6 mg Cu/kg diet than in fish in the two lowest (0.11 and 1.7 mg Cu/kg) and two highest copper groups (11 and 20 mg Cu/kg). Hepatic TBARS levels were lowest in fish fed the 4.4 and 6.6 mg Cu/kg diet, and highest in the 20 mg Cu/kg dietary group. Cu-Zn SOD activity was highest in fish fed the 6.6 mg Cu/kg diet, followed by the 9.0 mg Cu/kg dietary group, 20 mg Cu/kg dietary group, and lowest in the unsupplemented control group. Plasma ceruloplasmin was highest in fish fed the 6.6 mg Cu/kg diet, followed by the 2.4, 4.4 and 9.0 mg Cu/kg dietary groups, the 11 and 20 mg Cu/kg dietary groups, and lowest in the 0.11 and 1.7 mg Cu/kg dietary groups. The dietary copper requirement in growing grouper was found to be about 4–6 mg Cu/kg based on growth, hepatic Cu-Zn SOD activity, TBARS and whole-body copper retention.

The dietary copper requirement for grouper was re-evaluated by Lin et al. (2010), using an organic copper source (copper peptide). Lin et al. (2010) fed juvenile grouper, *Epinephelus malabaricus*, diets with 0.21, 2.0, 2.8, 3.8, 4.8, 9.9 or 14.1 mg Cu/kg DM for 8 weeks. Fish fed the diet containing 2.8 mg Cu/kg DM had the highest weight gain, followed by fish fed the 3.8 mg Cu/kg DM diet, and lowest in the 0.21, 9.9 and 14.1 mg Cu/kg DM dietary groups. Hepatic Cu-Zn SOD activity was highest in the 2.8 mg Cu/kg DM dietary group, followed by fish fed diets with 3.8, 9.9, 2.0 and 14.1 mg Cu/kg DM (in order of decreasing activity), and lowest in fish fed the diet containing 0.21 mg Cu/kg DM. TBARS was lowest in fish fed the 2.8 and 3.8 mg Cu/kg DM diets, followed by fish fed diets with 0.21 and 4.8 mg Cu/kg DM, and highest in fish fed the 14.1 mg Cu/kg DM diet. The dietary copper (as copper peptide) requirement for grouper was reported to be 2.4–3.7 mg Cu/kg DM based on weight gain, hepatic Cu-Zn SOD activity, TBARS and whole-body copper retention.

Juvenile yellow catfish (*Pelteobagrus fulvidraco*) were fed graded levels of dietary copper (2.3 (unsupplemented), 3.5, 4.9, 7.6, 13 or 24 mg Cu/kg DM) for 7 weeks (Tan et al., 2011). Fish fed the diets containing 3.5, 4.9 and 7.6 mg Cu/kg had significantly higher weight gain and SGR than the control fish, as well as fish fed 13 and 24 mg Cu/kg. The lowest feed conversion rate, protein efficiency ratio, hepatosomatic index and viscerosomatic index were observed in fish in the 24 mg Cu/kg dietary treatment. There were no significant differences in condition factor among the treatments. Whole-body and liver copper levels increased with dietary copper concentration, whereas muscle

[25] Daily weight gain expressed as a percentage of body weight.

copper content was relatively stable. The dietary copper requirement in juvenile yellow catfish was 3.4–4.6 mg/kg DW based on SGR and whole-body copper retention.

The effects of dietary copper on growth, digestive and absorptive enzyme activities, and antioxidant status in juvenile grass carp (*Ctenopharyngodon idella*) were investigated by Tang et al. (2013). Grass carp were fed diets containing 0.74 (control feed), 2.3, 3.8, 5.3, 6.7 and 8.3 mg Cu/kg diet wet weight for 8 weeks. Weight gain and feed efficiency increased with dietary copper concentrations up to 3.8 mg/kg. Activity of trypsin, chymotrypsin, and lipase, Na^+, K^+-ATPase, alkaline phosphatase, creatine kinase, γ-glutamyl transpeptidase superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and glutathione were significantly higher in fish fed the 3.8 and 5.3 mg Cu/kg diets. The dietary copper requirement of juvenile grass carp was between 4.7 and 5.0 mg/kg wet weight based on weight gain, feed efficiency and plasma ceruloplasmin activity.

Shao et al. (2012) investigated the effects of exposure to graded dietary levels of tribasic copper chloride (0, 3, 6, 9, 25, 50, 100 or 150 mg supplemental Cu/kg DM feed; analysed levels: 9, 12, 14, 18, 34, 57 103 or 148 mg total Cu/kg DM feed) for 56 days on growth, copper status and antioxidant activities in blunt snout bream (*Megalobrama amblycephala*). Weight gain and SGR were significantly higher in fish fed diets supplemented with the 3–6 mg Cu/kg DM feed than in fish fed the unsupplemented control diet. Fish fed the diet supplemented with the 9 mg Cu/kg DM feed had a significantly lower F/G than control fish. Significant copper accumulation in liver, kidney and intestine was evident in fish fed diets supplemented with the 50–150 mg Cu/kg DM feed. Dietary copper supplemented at levels ranging from 9 to 100 mg/kg DM feed significantly enhanced total antioxidant competence activity in liver, and fish fed diets supplemented with the 100–150 mg Cu/kg DM feed had significantly increased plasma alkaline and acid phosphatase activity. Diets with 12–14 mg total Cu/kg (supplemented with 3–6 mg Cu/kg DM feed) met the copper requirement for blunt snout bream; however, higher total copper (18 mg total Cu/kg DM feed; supplementation 9 mg Cu/kg DM feed) improved F/G in practical diets.

Juvenile large yellow croaker (*Larimichthys croceus*) were fed graded levels of dietary copper (2.8, 3.5, 5.1, 7.8, 12 or 20 mg Cu/kg DM) for 10 weeks to assess the dietary requirement (Cao et al., 2014). Fish fed the basal diet with 2.8 mg Cu/kg had the lowest weight gain rate (WGR), Cu-Zn SOD activity, total antioxidant capacity (T-AOC), whole-body and vertebrae copper concentrations. When dietary copper increased from 2.8 to 5.1 mg/kg, the WGR significantly increased ($p < 0.05$). Higher dietary copper contents did not result in further increase in WGR. Fish in the dietary groups with copper concentrations above 7.8 mg/kg had lower serum Cu-Zn SOD activity and T-AOC in liver. No significant differences were found in survival, feed efficiency, hepatosomatic index, viscerosomatic index or condition factor among treatments. The copper requirement for large yellow croaker was estimated to be 3.7 mg kg DM based on WGR, and 5.7–7.6 mg Cu/kg DM based on whole-body and vertebrae copper concentration and Cu-Zn SOD activity.

El Basuini et al. (2016) fed red sea bream (*Pagrus major*) diets containing 1.8 mg Cu/kg DW (control), 5.4 mg Cu/kg DW (4 mg/kg supplemented as copper sulphate), 3.5, 5.0, 7.9 or 9.7 mg Cu/kg DM (supplemented as 2, 4, 6 or 8 mg nanocopper (Sigma-Aldrich, 99% purity)/kg) for 60 days. Sea bream on diets supplemented with 2 and 4 mg copper nanoparticles/kg had the highest specific growth rate, protein gain and tolerance to low-salinity stress. Feed efficiency ratio, protein efficiency ratio, protein retention, whole-body copper content, protease activity, lysozyme activity and total serum protein were significantly higher in fish fed the diet supplemented with 2 mg copper nanoparticles/kg DW compared to the control group. Whole-body protein content of fish fed the control diet was significantly lower than that of fish fed copper-supplemented diets. The authors recommended adding 2 mg copper nanoparticles/kg diet to improve growth, immune response and antioxidant defence system in red sea bream.

Juvenile Russian sturgeon (*Acipenser gueldenstaedtii*) were fed diets containing 0.33, 1.7, 2.7, 4.9, 6.9, 9.2 or 17.9 mg Cu/kg DM for 8 weeks (Wang et al., 2016). The WGR and feed efficiency were significantly higher in fish fed 2.7–9.2 mg Cu/kg DM than in the control group, 1.7 or 17.9 mg Cu/kg DM dietary treatments. The whole-body Cu concentrations were significantly lower in the fish fed 0.33, 1.7, 2.7 or 4.9 mg Cu/kg DM than in fish fed 6.9–17.9 mg Cu/kg DM. Hepatic Cu-Zn SOD activity, total antioxidant capacity and serum ceruloplasmin activity were significantly higher in fish in the 6.9 and 9.2 mg Cu/kg DM dietary treatments than in the other groups. The lowest hepatic TBARS values were found in fish fed 6.9 and 9.2 mg Cu/kg diets. No differences were observed in survival, hepatosomatic index, viscerosomatic index or condition factor among the treatments. The dietary copper requirement for juvenile Russian sturgeon was established to 6.8–8.2 mg/kg DM based on the WGR, whole-body Cu concentration, hepatic Cu-Zn SOD and serum ceruloplasmin activity.

Appendix B – Summary output of the polynomial regression fit calculated for data in Figure 2

Polynomial regression 3 order ($y = 28.0048 + 0.1032x - 0.0011x^2 + 0.00005x^3$; $R^2 = 0.6868$)

Regression statistics	
Multiple *R*	0.828764782
R^2	0.686851064
Adjusted R^2	0.670928237
Standard error	94.15717122
Observations	63

ANOVA

	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	1,147,282.72	382,427.573	43.1362505	6.9043E-15
Residual	59	523,068.801	8,865.57289		
Total	62	1,670,351.52			

	Coefficients	**Standard error**	**t stat**	**p-value**	**Lower 95%**	**Upper 95%**
Intercept	28.00481785	38.2419239	0.73230672	0.4668806	−48.517095	104.526731
Cu diet	0.103231774	1.50802014	0.06845517	0.94565478	−2.91430955	3.1207731
Cu_2 diet	−0.002174216	0.01269474	−0.17126897	0.86459833	−0.02757634	0.02322791
Cu_3 diet	2.73105E-05	2.9031E-05	0.94074393	0.35067197	−3.078E-05	8.5401E-05

Appendix C – Data of copper in compound feed from monitoring activities in European countries

Validation procedure:

- The first criterion consisted in the inclusion/exclusion of samples considering the type of feed. For most animal species/categories, only *Complete feed* samples were considered. For bovids and horses, both complete and complementary feed were considered; for *Complementary feed*, samples labelled as 100% of the daily ration were attributed to complete feed. Dog and cat wet food samples were excluded as the dry matter content was not reported.
- As a second criterion, the content of copper was taken. Descriptive parameters of data distribution would be biased by unlikely low copper concentrations as well as by excessive levels which may be driven by intentions other than the production of standard feed (e.g. disease prevention). Therefore, copper concentrations below 5 mg/kg (minimum background level (except milk replacer); see Section 3.3.2) were not considered. Maximum cut-off values were built for complete feed (i) with copper concentrations exceeding the requirements (in all cases 10 mg/kg) by a factor of 4; (ii) sheep and calves milk replacer were considered sensitive species and the maximum copper content (15 mg/kg feed) plus the 20% tolerance (see Regulation 767/2009) was taken (18 mg/kg); (iii) for piglets, the maximum currently authorised copper concentration in feed (170 mg) plus the 20% tolerance (see Regulation 767/2009) was considered (205 mg/kg).

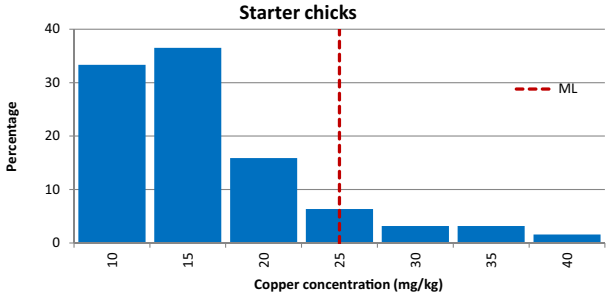
Table C.1: Data submitted from European Countries as response to a questionnaire on 'Analyses of Compound Feed for all animal species/categories obtained during National Official Controls': raw data, data validation and analysed data

Compound feed	All data	Excluded data based on feed type	Excluded data based on copper amount	Data analysed
Poultry	1,283	77	50	1,156
Starter chicks	71	0	8	63
Laying hens	529	67	22	440
Chickens for fattening	387	8	19	360
Turkeys	204	2	0	202
Other poultry: ducks and geese	92	0	1	91
Pigs	4,750	399	351	4,000
Piglets (Weaned, Starter I, Starter II)	1,545	87	38	1,420
Pigs for fattening (Fattening, Grower, Finisher)	2,529	266	229	2,034
Sows	676	46	84	546
Bovids	3,060	2,785	53	222
Calf milk replacer	502	376	15	111
Fattening cattle	862	802	18	42
Dairy cow	1,182	1,148	3	31
Sheep	464	418	16	30
Goat	50	41	1	8
Horses	346	343	1	2
Rabbits	217	13	2	202
Fish	41	1	0	40
Dogs	213	11	71	131
Cats	82	5	24	53
Total	9,992	3,634	552	5,806

Appendix D – Copper in feed (mg/kg)

Figures and Tables D.1–D.17: Data from European countries (years 2012–2014)

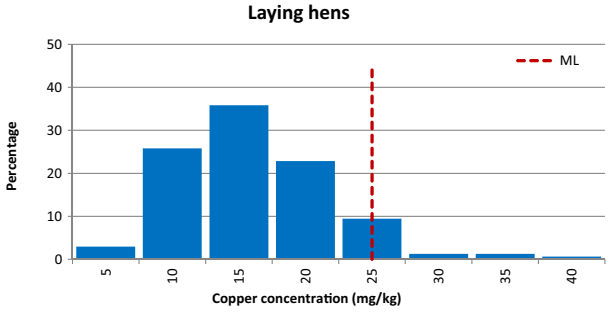
Figure and Table D.1: Starter chicks



Number of samples	63
Mean of copper in feed (mg/kg)	16
P10	10
Median	14
P90	25
Range used	5–40
% samples above limit (25)	7.9
% samples below limit (25)	92.1

Number of samples per country: CZ: 28; EE: 2; DE: 1; IT: 30; LU: 1; UK: 1.

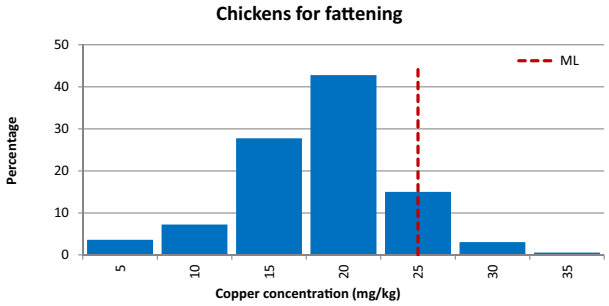
Figure and Table D.2: Laying hens



Number of samples	440
Mean of copper in feed (mg/kg)	16
P10	9
Median	15
P90	23
Range used	5–40
% samples above limit (25)	5.9
% samples below limit (25)	94.1

Number of samples per country: BE: 4; CY: 1; CZ: 119; DE: 233; EE: 2; GR: 4; HU: 12; IT: 28; LU: 18; SI: 10; UK: 9.

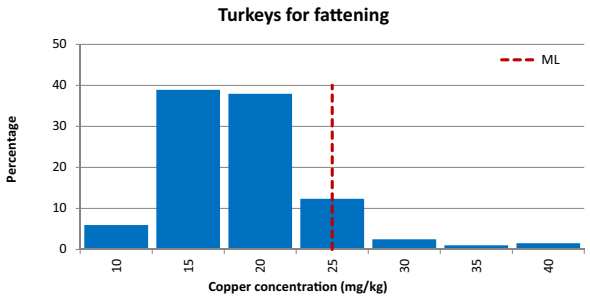
Figure and Table D.3: Chicken for fattening



Number of samples	360
Mean of copper in feed (mg/kg)	18
P10	12
Median	19
P90	24
Range used	10–40
% samples above limit (25)	7.2
% samples below limit (25)	92.8

Number of samples per country: BE: 10; CY: 1; CZ: 110; DE: 144; GR: 8; HU: 27; IT: 50; LU: 3; SI: 7.

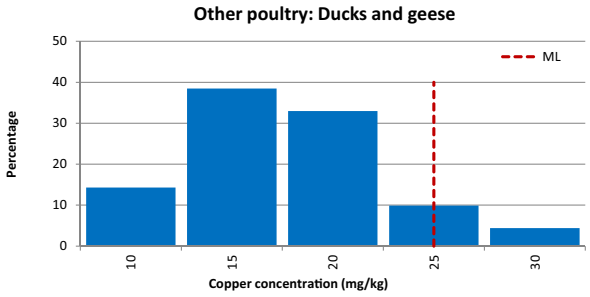
Figure and Table D.4: Turkeys for fattening



Number of samples	203
Mean of copper in feed (mg/kg)	19
P10	14
Median	18
P90	24
Range used	5–40
% samples above limit (25)	7.9
% samples below limit (25)	92.1

Number of samples per country: CZ: 20; DE: 146; HU: 10; IT: 23; LU: 1; SI: 1; UK: 2.

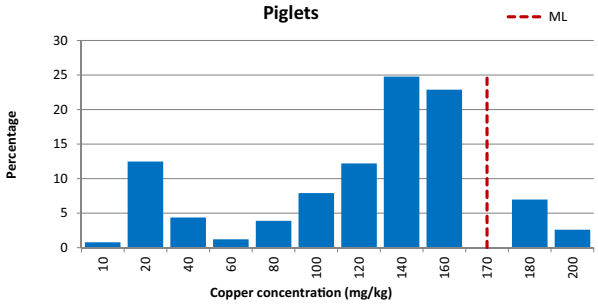
Figure and Table D.5: Other poultry: duck and geese



Number of samples	91
Mean of copper in feed (mg/kg)	17
P10	12
Median	17
P90	23
Range used	5–40
% samples above limit (25)	4.4
% samples below limit (25)	95.6

Number of samples per country: CZ: 26; DE: 57; HU: 7; UK: 1.

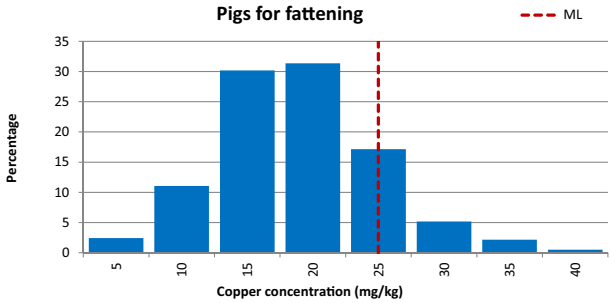
Figure and Table D.6: Piglets



Number of samples	1,420
Mean of copper in feed (mg/kg)	119
P10	23
Median	136
P90	168
Range used	5–205
% samples above limit (170)	8.8
% samples below limit (170)	91.2

Number of samples per country: BE: 43; CY: 1; CZ: 50; DE: 1030; EE: 31; GR: 17; HU: 199; IT: 16; LU: 27; SI: 3; UK: 3.

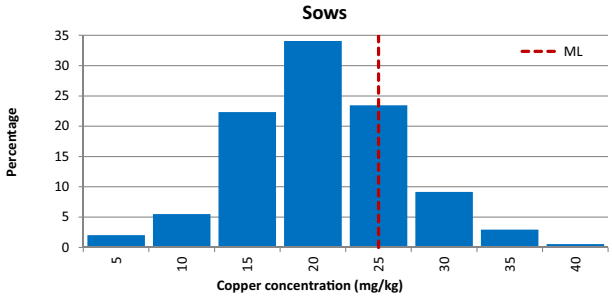
Figure and Table D.7: Pigs for fattening



Number of samples	2,034
Mean of copper in feed (mg/kg)	19
P10	12
Median	18
P90	26
Range used	5–40
% samples above limit (25)	12.9
% samples below limit (25)	87.1

Number of samples per country: BE: 15; CY: 8; CZ: 356; DE: 1102; EE: 32; GR: 26; HU: 255; IT: 62; LU: 19; PT: 116; SI: 30; UK: 13.

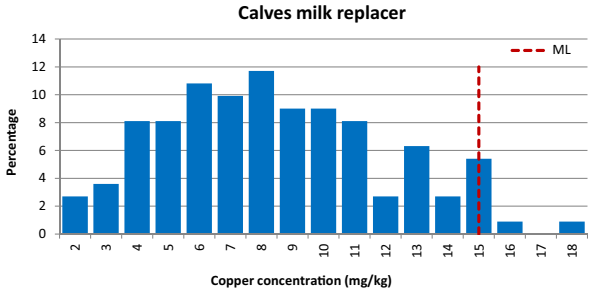
Figure and Table D.8: Sows



Number of samples	546
Mean of copper in feed (mg/kg)	21
P10	13
Median	20
P90	29
Range used	5–40
% samples above limit (25)	20.1
% samples below limit (25)	79.9

Number of samples per country: BE: 3; CZ: 79; DE: 345; EE: 8; GR: 6; HU: 53; IT: 7; LU: 15; PT: 16; SI: 6; UK: 8.

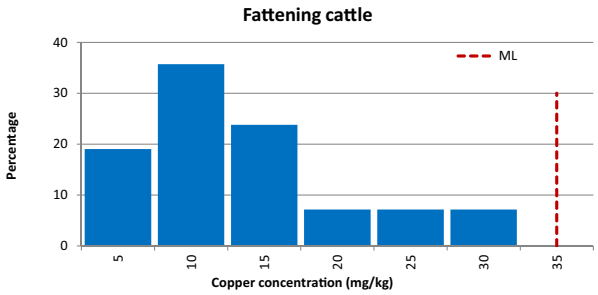
Figure and Table D.9: Calves milk replacer



Number of samples	111
Mean of copper in feed (mg/kg)	8
P10	4
Median	8
P90	13
Range used	2–18
% samples above limit (15)	3.6
% samples below limit (15)	96.4

Number of samples per country: BE: 2; CZ: 19; DE: 78; LU: 9; SI: 3.

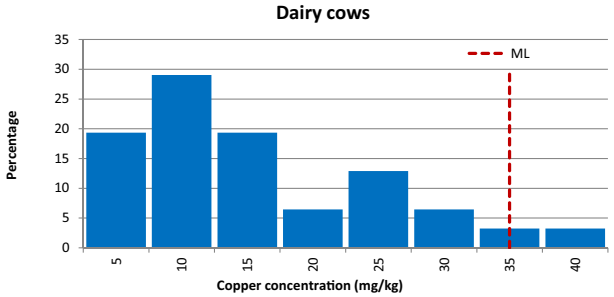
Figure and Table D.10: Fattening cattle



Number of samples	42
Mean of copper in feed (mg/kg)	13
Median	12
Range used	5–40
% samples above limit (35)	0
% samples below limit (35)	100

Number of samples per country: DE: 32; SI: 1; UK: 9.

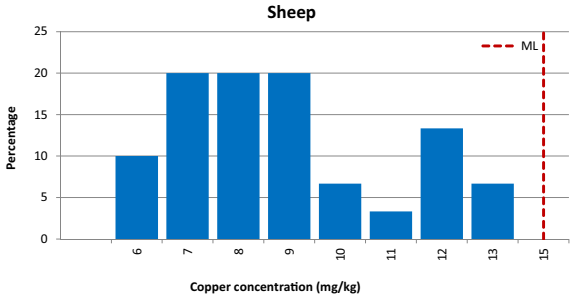
Figure and Table D.11: Dairy cows



Number of samples	31
Mean of copper in feed (mg/kg)	16
Median	13
Range used	5–40
% samples above limit (35)	6.5
% samples below limit (35)	93.5

Number of samples per country: CZ: 7; DE: 15; EE: 1; GR: 1; UK: 7.

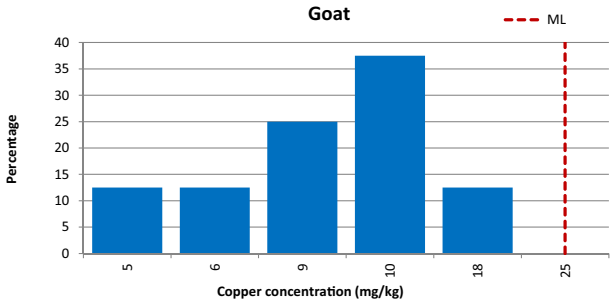
Figure and Table D.12: Sheep



Number of samples	30
Mean of copper in feed (mg/kg)	9
Median	8
Range used	5–18
% samples above limit (15)	0
% samples below limit (15)	100

Number of samples per country: DE: 9; GR: 15; HU: 3; UK: 3.

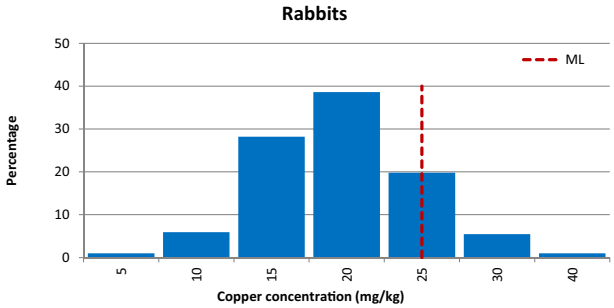
Figure and Table D.13: Goat



Number of samples	8
Mean of copper in feed (mg/kg)	9
Median	9
Range used	5–40
% samples above limit (25)	0
% samples below limit (25)	100

Number of samples per country: GR: 7; UK: 1.

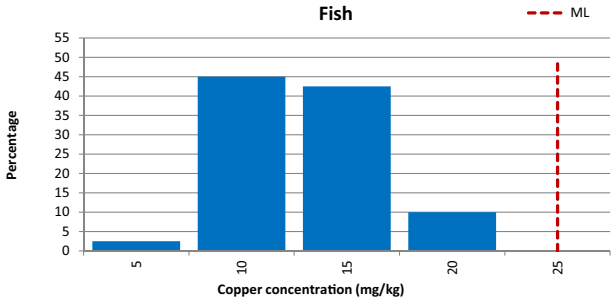
Figure and Table D.14: Rabbits



Number of samples	202
Mean of copper in feed (mg/kg)	20
P10	14
Median	19
P90	25
Range used	5–40
% samples above limit (25)	10.4
% samples below limit (25)	89.6

Number of samples per country: BE: 2; CZ: 78; DE: 116; HU: 3; IT: 1; SI: 2.

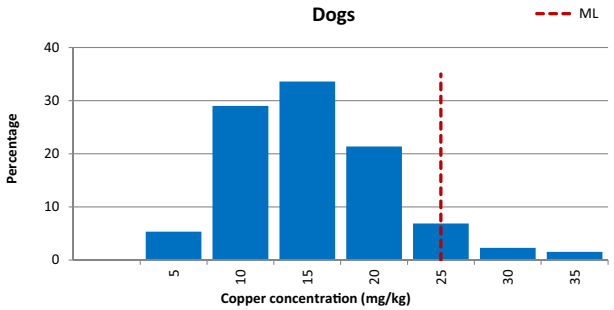
Figure and Table D.15: Fish



Number of samples	40
Mean of copper in feed (mg/kg)	13
Median	13
Range used	5–40
% samples above limit (25)	0
% samples below limit (25)	100

Number of samples per country: CZ: 1; DE: 32; EE: 1; GR: 3; UK: 3.

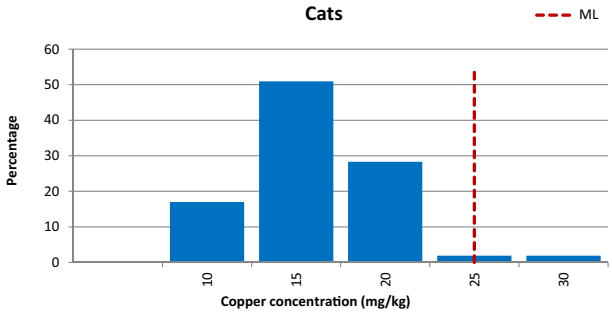
Figure and Table D.16: Dogs



Number of samples	131
Mean of copper in feed (mg/kg)	15
P10	10
Median	14
P90	23
Range used	5–40
% samples above limit (25)	6.9
% samples below limit (25)	93.1

Number of samples per country: CZ: 79; DE: 25; HU: 16; SI: 4; UK: 7.

Figure and Table D.17: Cats



Number of samples	53
Mean of copper in feed (mg/kg)	16
P10	12
Median	17
P90	21
Range used	5–40
% samples above limit (25)	1.9
% samples below limit (25)	98.1

Number of samples per country: CZ: 38; DE: 11; SI: 2; UK: 2.

Table D.18: Data from Norwegian Seafood Safety Authority's Annual Monitoring Programme (2000–2014) by the National Institute of Nutrition and Seafood Research (NIFES)

Copper in fish feed (for salmon and rainbow trout, mg/kg feed DM). From: Sissener et al. (2013) and Sanden et al. (2014, 2015)

Year	Samples (n)	Mean	Range
2000	83	11.6	5.4–18.0
2001	23	13.5	9.7–14.6
2002	33	9.3	4.4–15.6
2003	39	9.7	2.7–15.3
2004	40	10.9	5.3–21.0
2005	23	8.7	2.5–15.0
2006	49	9.9	2.6–17.0
2007	22	10.5	3.1–16.0
2008	21	13.3	8.6–21.0
2009	25	10.1	8.0–13.0
2010	23	9.7	4.7–15.0
2011	25	9.6	4.9–15.5
2012	23	10.1	5.9–17.0
2013	69	9.2	5.1–18.0
2014	73	10	5.0–65.0

Appendix E – Specifications of the literature search: influence of copper in the development of antimicrobial resistance of bacteria in the environment

A literature search was conducted to answer the review question 'Does the concentration of copper in soil trigger antibiotic resistance in soil bacteria?'

The literature was searched in three databases (AGRIS, ISI Web of Knowledge and PubMed) on 9 February 2016. The following search string was used: copper OR Cu OR copper sulphate AND soil OR environment AND antibiotic resistance OR antimicrobial resistance.

A total of 574 records were collected from the three databases and included in an EndNote Library. Four references collected from European countries and four references cited in the Chapter in the previous Scientific Opinion on copper[26] were also included. Elimination of duplicates resulted in 556 remaining references. After Title and Abstract screening, the references were classified into Appropriate, Doubtful or Excluded. The distribution was as follows: 93 Appropriate, 46 Doubtful and 417 Excluded.

An additional review aiming to reduce the number of *Appropriate* references was done. Three criteria were proposed for such review: (a) papers published since 2011 and onwards; (b) dealing simultaneously with Copper and Soil/Environment; and (c) studies conducted in Europe. (The last criterion was, in the end, not applied, because of the low number of studies remaining). After this review, 41 references were selected. A revision of the papers was conducted and they were allocated to one of the following six categories:

- Aquatic environment polluted with antibiotics and heavy metals
- Correlation between heavy metals and antibiotic resistance
- Co-transfer of heavy metal and antibiotic resistance
- Manuring
- Copper and antibiotic resistance in sediments
- Copper and antibiotic resistance in wastewater

[26] EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Scientific Opinion on the safety and efficacy of copper compounds (E4) as feed additives for all animal species: cupric sulphate pentahydrate based on a dossier submitted by Manica S.p.A. EFSA Journal 2012;10(12):2969, 38 pp. doi:10.2903/j.efsa.2012.2969

Appendix F – Calculations derived from data of pigs and bovids feed to obtain estimations of savings of copper emissions to the environment

Target species, animal category	CAMC	NPMC	Reduction (mg/kg)	EU compound feed production in 2015 (×1,000 tonnes)	Corrected compound EU feed production estimated from MS with missing category information (×1,000 tonnes)	Quantity of copper saved from going into fields (tonnes/ year)
Piglet, weaned	170	25	145	4,308	5,980	868
Cattle for fattening	35	30	5	7,401	10,900	54
Dairy cow	35	30	5	17,155	22,600	113
Goat	25	35	−10	849		−8

Annex A – Examples of copper enzymes

Enzymes	Function	Comments
Amine oxidases Benzylamine oxidase Diamine oxidase Indole 2,3-dioxygenase Spermine oxidase	Deamination of primary amines	Present in all eukaryotes; catalyse the oxidation of biogenic amines (e.g. tyramine, histidine and polyamines) producing oxidised organic products, usually aldehydes, and generating NH_3 and H_2O_2 as by-products
Ceramide glactosyl transferase	Myelin synthesis	Responsible for synthesis and maintenance of phospholipid membranes. Ganglioside lipid components (ceramide) are synthesised in the endoplasmic reticulum and glycosylated by glycosyltransferase in the Golgi network compartments
Multi-copper ferroxidases Ceruloplasmin, GPI-ceruloplasmin, hephaestin, zyklopen Hephaestin Ferroxidase for cellular export of iron Ceruloplasmin Plasma metallo/ferroxidase	Copper transport, oxidation Iron trafficking	Multicopper oxidase containing 65–90% of vertebrate serum copper; six tightly bound copper ions at two distinct molecular sites; one appears to activate oxygen; the other appears to be involved in electron transport. CP likely major copper transport molecule in higher mammals by binding to cellular membrane receptors and releasing Cu Aceruloplasmic individuals (humans and knockout mice) show severe difficulties with iron overload in central nervous system (CNS); deficiency may manifest as neurological damage but minimal (if any) difficulties with Cu metabolism
Cytochrome *c* oxidase	Electron transport, terminal oxidase	Mitochondrial oxidative phosphorylation transfer of electron to O_2 Terminal enzyme in the respiratory chain, located in the inner membrane of mitochondria and bacteria Catalyses the reduction of O_2 to water; pumps one proton across membrane for each proton consumed in the reaction to generate electro-chemical gradients that can be used for other cellular purposes (e.g. ATP synthesis). Deficiency may lead to brain abnormalities, hypothermia, muscle weakness
Dopamine-β-hydroxylase	Norepinephrine and epinephrine synthesis	Catalyses conversion of dopamine to norepinephrine. Deficiency may lead to neurological effects, possible hypothermia
Lysyl (and hydroxylysine) oxidase	Cross-linking of collagen and elastin	Secreted enzyme catalysing the deamination of peptidyl lysine residues forming inter- or intrachain covalent cross-links in elastin and collagens. Critical extracellular matrix integrity lung, bone matrix, cardiovascular integrity. Found in the nucleus where it may function in chromatin organisation
Monoamine oxidase	Neurotransmitter synthesis	Found predominantly on the outer mitochondrial membrane throughout the CNS. Two isozymes both of which oxidise the neurotransmitters dopamine, epinephrine, tyramine and tryptamine, although monoamine oxidase (MAO)-A selectively oxidises serotonin; MAO-B oxidises phenylethylamine and benzylamine. Deficiencies of MAO-A result in disturbed systemic amine metabolism, borderline mental retardation and possible cardiovascular and behavioural abnormalities. Monoamine oxidase inhibitors
Peptidylglycine α-amidating mono-oxygenase	Neuropeptide processing	Multifunctional protein involved in the maturation of bioactive hormones (e.g. neurotransmitters and growth hormones) containing two enzymes that act sequentially to catalyse the alpha-amidation of neuroendocrine peptides Melanocyte-stimulating hormone Degradation of amines, e.g. serotonin, catecholamines, dopamine and tyramine

Enzymes	Function	Comments
Polyphenol oxidase/ tyrosinase	Quinone biosynthesis Pigment (melanin) synthesis; amino acid metabolism	Tyrosine→dopa→dopaquinone→melanin production Polyphenol oxidase oxidises tyrosine to dihydroxyphenylalanine which in turn is oxidised to *o*-quinone Oxidises tyrosine to the pigment melanin in mammals. Deficiency leads to hypopigmentation
Prostaglandin reductase	Prostaglandin biosynthesis	Responsible for reduction of 15-oxoprostaglandins to 13,14-dihydro derivatives
Uricase	Nucleic acid metabolism	Hepatic and renal metabolism of uric acid Termination of purine catabolism Also found in tissues of lower mammals and in some plants and microorganisms
(Cu-Zn) superoxide dismutase	Destruction of superoxide radicals	Superoxide conversion Cytosolic antioxidant; $2O^{-}_{2} + 2H^{+} \leftrightarrows H_2O_2 + O_2$ Deficiency results in sensitivity to oxidative stress
Diamine oxidase Tryptophan 2,3-dioxygenase Indole 2.3-dioxygenase		Degrades histamine and polyamines Tryptophan to *N*-formyl-l-kynurenine Indole to 2-formylaminobenzaldehyde
Thiol oxidase		Formation of disulphide linkages
Ascorbate oxidase	Terminal oxidase	Found in a wide variety of fruits and vegetables, including Valencia oranges, cucumbers and zucchini. Couples one-electron oxidation of ascorbate to full molecular reduction of O_2 to water
Galactose oxidase	Carbohydrate metabolism	Fungi: Catalyse the oxidation of primary alcohols to their corresponding aldehydes; hydrogen peroxide often produced. In fungi, may be involved in breaking down the plant cell wall prior to invasion
Haemocyanin	Oxygen transport	Blue respiratory protein (oxygen carrier) of decapod crustaceans such as lobster, crabs and crayfish (contrast with iron-containing blood pigments such as haemoglobins (red), myoglobins (red), chlorocruorins (green) and haemerythrins (violet)
Stellacyanin	Electron transport; superoxide radicals	Found in fungi and some plants including the sap of Japanese lacquer trees, cucumber peel, horseradish roots, and *Arabdiopsis thaliana* Appear to be elements of cell wall, and may be involved with oxidative cross-linking reactions, along with peroxidases, ascorbic acid oxidase and laccase
Rusticyanin	Electron transport	Found in fungi and Gram-negative bacteria. Can tolerate extremes of pH
Plastocyanin	Electron transfer in plants	Small (10.5 kDa) protein functioning as an electron carrier between the cytochrome *b6f* photosystem 1 (PS 1) complexes in the photosynthetic electron-transfer chain
Laccase	Terminal oxidase	Found in plants and fungi. Catalyses the four-electron reduction of O_2 to H_2O_2 and depolymerises lignin

Reference

Stern BR, Solioz M, Krewski D, Aggett PJ, Aw T-C, Baker S, Crump K, Dourson M, Haber L, Hertzberg R, Keen C, Meek B, Rudenko L, Schoeny R, Slob W and Starr T, 2007. Copper and Human Health: Biochemistry, Genetics, and Strategies for Modeling Dose Response Relationships. Journal of Toxicology and Environmental Health, Part B. Critical Reviews, 10, 157–222.

Annex B – Copper concentration in feed materials

Table B.1 (I): Copper concentration in feed materials according to CVB[(a)] feed composition tables (in mg/kg feed material *as is*) and in mineral feed materials according to Batal and Dale (2008)[(b)]

Feed materials	mg/kg	Feed materials	mg/kg
Alfalfa meal	6–9	Barley	4
Barley feed (residue of polishing)	9	Barley milling by-product	6
Beans (phaseolus) heat treated	9	Biscuits	2–3
Blood meal spray dried	11	Bone meal	4
Bread meal	1	Brewers' grains dried	20
Brewers' yeast dried	9	Buckwheat	10
Canary seed	5	Carob	3
Casein	1	Chicory pulp dried	6
Citrus pulp dried	5	Coconut expeller	29–32
Coconut extracted	31	Cotton expeller with hulls	16
Cotton extracted with hulls	15	Distillers grains and solubles	5
Fat from animals	2	Feather meal hydrolysed	11
Fish meal	6–8	Grass meal	8
Horsebeans	13	Horsebeans white	13
Lentils	10	Linseed	12
Linseed expeller	18	Linseed extracted	17
Lupins	5–6	Maize	1
Maize chemically-heat treated	2	Maize feed meal extracted	3
Maize feed flour	2	Maize germ meal expeller/extracted	5–7
Maize gluten feed	5–6	Maize gluten meal	7
Malt culms	13	Meat and bone meal	8–9
Meat meal	15–50	Milk powder skimmed	1
Milk powder whole	5	Millet	6
Nigerseed	13	Oats grain	4
Oats grain peeled	3	Oats husk meal	2
Palm kernels	13	Palmkern expeller	23–24
Peanut expeller	21	Peanut extracted	33–34
Peas	7	Potatoes sweet dried	6
Potato pulp	6	Potato starch	1
Potato protein	23–33	Rapes meal	4
Rapeseed	3	Rapeseed expeller	7
Rapeseed extracted	5–6	Rice bran meal extracted	11
Rice feed	7–8	Rice with hulls	1
Rye	3	Sesame seed meal extracted	43
Soybean meal	11–15	Sorghum	3
Soybean hulls	8	Soybean expeller	18
Sugarbeet pulp	4–8	Soybeans	12
Sunflowers with hulls/dehulled	14	Sugarbeet/sugarcane molasses	6–9
Sunflower meal	28–36	Sunflower expeller with hulls/dehulled	28
Triticale	5	Tapioca	2–4
Wheat	3	Vinasse sugarbeet	8
Wheat feed meal	11	Wheat bran	31
Wheat germ	10	Wheat feed flours	6
Wheat gluten meal	6	Wheat germ feed	10
Wheat middlings	10	Wheat gluten feed	7
Whey powder partially delactosed	3	Whey powder	1

(a): Centraal Veevoederbureau (CVB). 2009. Feed Tables. Produktschap Diervoeding, the Netherlands.
(b): Batal and Dale. 2008. Feedstuffs September 10, p. 16.

Table B.1 (II): Copper concentration in feed materials according to CVB[(a)] feed composition tables (in mg/kg DM) and in mineral feed materials (in mg/kg *as is*) according to Batal and Dale (2008)[(c)]

Moisture-rich feed materials	mg/kg DM	Moisture-rich feed materials	mg/kg DM
Beet pulp fresh/ensiled	5	Wheat starch	6–7
Brewers yeast	15	Brewers grains	7–16
Whey	13–19	Corn cob meal	2–3
Maize gluten feed fresh/ensiled	5	Chicory pulp fresh/ensiled	10
Potato starch, different products	10–15	Maize solubles	14
Potato juice concentrate	47	Potato pulp	4–6
Roughages and comparable products	**mg/kg DM**	**Roughages and comparable products**	**mg/kg DM**
Beet leaves ensiled	10	Cucumber fresh	2
Clover red silage	11	Gras, average	8–9
Gras silage, average	8	Green cereals silage	6
Lucerne (alfalfa) ad[(b)]	9	Lucerne silage	9
Maize (fodder) ad[(b)]	4	Maize silage	4
Maize, fresh	4	Whole crop silage (cereals)	5
Mineral feed materials	**mg/kg**	**Mineral feed materials**	**mg/kg**
Bone meal (steamed)	16	Diammonium phosphate	80
Difluorinated phosphate	22	Dicalcium phosphate	80
Mono-dicalcium phosphate	70	Monoammonium phosphate	80
Calcium carbonate/limestone	24		

(a): Centraal Veevoederbureau (CVB). 2009. Feed Tables. Produktschap Diervoeding, the Netherlands.
(b): ad: air dried.
(c): Batal and Dale. 2008. Feedstuffs September 10, p. 16.

Table B.2: Copper concentration in feed materials according to INRA[(a)] feed composition tables (in mg/kg feed material *as is*)

Cereals	mg/kg ± SD	Cereals	mg/kg ± SD
Barley	9 ± 5	Maize	2 ± 1
Oats	3 ± 1	Oats groats	3
Rice, brown	2	Rye	5
Sorghum	4 ± 2	Triticale	6 ± 3
Wheat, durum	7	Wheat, soft	5 ± 10
Wheat by-products	**mg/kg ± SD**	**Wheat by-products**	**mg/kg ± SD**
Wheat bran	17 ± 25	Wheat middlings	12 ± 2
Wheat shorts	14	Wheat feed flour	6
Wheat gluten feed, starch 25%	7	Wheat gluten feed, starch 28%	7
Maize by-products	**mg/kg ± SD**	**Maize by-products**	**mg/kg ± SD**
Corn distillers	10	Corn gluten feed	5 ± 4
Corn gluten meal	11 ± 4	Maize bran	3
Maize germ meal, solvent extracted	12	Hominy feed	7
Other cereal by-products	**mg/kg ± SD**	**Other cereal by-products**	**mg/kg ± SD**
Barley rootlets, dried	10	Brewers' dried grains	18 ± 5
Rice bran, extracted	14	Rice bran, full fat	7 ± 4
Rice, broken	1		

Legume and oil seeds	mg/kg ± SD	Legume and oil seeds	mg/kg ± SD
Chickpea	6	Cottonseed, full fat	10 ± 1
Faba bean, coloured flowers	12 ± 2	Faba bean, coloured flowers	11
Linseed, full fat	12	Lupin, blue	5
Lupin, white	4	Pea	7 ± 1
Rapeseed, full fat	3	Soybean, full fat, extruded	3
Soybean, full fat, toasted	3	Sunflower seed, full fat	2
Oil seed meals	**mg/kg ± SD**	**Oil seed meals**	**mg/kg ± SD**
Copra meal, expeller	32	Cottonseed meal, CF 7–14%	19
Cottonseed meal, CF 14–20%	10	Grapeseed oil meal, solvent extracted	21
Groundnut meal, detoxified, CF < 9%	17	Groundnut meal, detoxified, CF > 9%	15
Linseed meal, expeller	18	Linseed meal, solvent extracted	19
Palm kernel meal, expeller	21	Rapeseed meal	9
Sesame meal, expeller	34	Soybean meal	18 ± 7
Sunflower meal, partially decorticated	62	Sunflower meal, undecorticated	27
Other plant by-products	**mg/kg ± SD**	**Other plant by-products**	**mg/kg ± SD**
Beet pulp, dried	5 ± 2	Beet pulp dried, molasses added	4
Beet pulp, pressed	1	Brewers' yeast, dried	47
Carob pod meal	3	Citrus pulp, dried	3
Grape marc, dried	75	Liquid potato feed	2
Molasses, beet	13	Molasses, sugarcane	29
Potato protein concentrate	38	Potato pulp, dried	7
Soybean hulls	8 ± 3	Vinasse, from yeast production	9
Dehydrated forages	**mg/kg**	**Dehydrated forages**	**mg/kg ± SD**
Alfalfa, dehydrated, protein	5–9	Grass, dehydrated	7 ± 1
Wheat straw	3		
Dairy products	**mg/kg**	**Dairy products**	**mg/kg**
Milk powder, skimmed	3	Milk powder, whole	2
Whey powder, acidic	2	Whey powder, sweet	2
Fish meals and solubles	**mg/kg ± SD**	**Fish meals and solubles**	**mg/kg ± SD**
Fish meal, protein 62%	9	Fish meal, protein 65%	7 ± 1
Fish meal, protein 70%	7	Fish solubles, condensed, defatted	36
Other animal by-products	**mg/kg ± SD**	**Other animal by-products**	**mg/kg ± SD**
Blood meal	5 ± 0	Feather meal	9 ± 1
Meat and bone meal, fat < 7.5%	20	Meat and bone meal, fat > 7.5%	20

(a): INRA, 2004. Tables of composition and nutritional value of feed materials. Wageningen Academic Publishers, the Netherlands & INRA, Paris, France.

Table B.3: Copper concentration (mg/kg DM) in various cereal grains (Rodehutscord et al. 2016)

Cereal grains	Genotypes (n)	Mean	Range
Barley	21	5.01	4.27–6.20
Maize	27	2.04	1.04–4.11
Oats	11	3.64	3.15–4.25
Rye	22	4.26	3.74–4.87
Triticale	21	4.94	4.24–6.42
Wheat	29	4.27	3.59–5.42

Reference

Rodehutscord M, Rückert C, Maurere HP, Schenkel H, Schippreck W, Bach-Knudsen KE, Schollenberger M, Laux M, Eklund M, Siegert W and Mosenthin R, 2016. Variation in chemical composition and physical characteristics of cereal grains from different genotypes. Archives of Animal Nutrition, 70, 87–107.

Table B.4: Copper content (mg/kg DM) in grass silages from Baden-Württemberg (Germany) from 1995–2004 and 2008–2013 (Leberl et al., 2005; Schenkel, 2014)

Year	Cut	Number of samples	Copper
1995	1st	37	6.8[a]
	2nd ff	19	7.7[b]
1996	1st	261	7.3
	2nd ff	60	7.6
1997	1st	225	7.0
	2nd ff	70	7.2
1998	1st	202	7.5[a]
	2nd ff	97	7.9[b]
1999	1st	196	8.3
	2nd ff	49	8.8
2000	1st	209	8.4[a]
	2nd ff	72	9.1[b]
2001	1st	110	8.2
	2nd ff	88	8.3
2002	1st	283	7.0[a]
	2nd ff	122	7.6[b]
2003	1st	224	7.2[a]
	2nd ff	70	7.8[b]
2004	1st	151	8.0[a]
	2nd ff	83	6.8[b]
2008	1st	90	7.1 (4.6–12.4)
	2nd	70	7.7 (6.1–11.4)
	3rd	32	8.4 (6.6–10.7)
	4th	12	9.1 (7.7–10.4)
2009	1st	67	8.2 (3.8–10.4)
	2nd	35	8.9 (3.8–11.9)
	3rd	13	9.8 (7.9–11.6)
	4th	11	9.4 (7.6–10.7)
2011	1st	80	9.2 (6.0–15.0)
	2nd	28	9.7 (7.1–11.7)
2012	1st	61	8.3 (5.1–12.1)
	2nd	17	8.9 (6.1–12.2)
2013	1st	53	7.3 (5.1–12.0)
	2nd	19	6.8 (4.0–8.3)

ff: following

[a,b]: Different superscripts within a year indicate significant differences between cuts ($p < 0.05$).

References

Leberl P, Kiefer S and Schenkel H, 2005. Comparison of composition of grass silages in Baden-Wuerttenberg (Germany) between 1995–2004 (in German) Proceedings 14th Conference on Nutrition of Farm Animals, Zadravec-Erjavec (Slovenia) 14.–15.11.2005, pp. 126–132.

Schenkel H, 2014. Annual Reports of the County Research Station of Agricultural Chemistry, University Hohenheim (Germany).

Annex C – Background concentration of copper in complete feed, from several sources

Table C.1: Background concentration of copper in a representative complete feedingstuff for a list of animal species/categories using CVB[(a)] and INRA[(b)] trace element composition tables[(c)]

	Number of feed materials in the formula	Total copper background concentration (mg/kg as such) in complete feedingstuff	
		CVB	INRA[(d)]
Starter chicks (complete feed)	15	8.4	9.4
Chicken reared for laying (complete feed)	17	9.2	8.8
Layer Phase I (complete feed)	16	9.1	12.0
Layer Phase II (complete feed)	16	9.8	11.7
Broiler Starter (complete feed)	14	6.9	11.6
Broiler Grower (complete feed)	15	6.9	10.6
Broiler Finisher (complete feed)	15	6.8	10.6
Turkey Starter (complete feed)	14	9.5	11.0
Turkey Grower (complete feed)	13	9.5	11.7
Turkey Finisher (complete feed)	11	8.8	10.5
Turkey Breeder (complete feed)	8	5.3	6.1
Duck, grower/finisher (complete feed)	10	6.2	8.1
Geese, grower/finisher (complete feed)	8	6.9	8.9
Piglet Starter I (from weaning)	9	5.4	11.2
Piglet Starter II (complete feed)	20	6.1	7.8
Pig Grower (complete feed)	19	5.8	7.8
Pig Finisher (complete feed)	18	6.5	8.4
Sows, gestating (complete feed)	18	10.4	10.2
Sows, lactating (complete feed)	20	7.5	9.2
Calf, milk replacer (complete feed)	10	4.8	5.0
Calf (complete feed)	17	8.4	9.1
Calf concentrate (complementary feed)	16	8.9	11.3
Cattle (complete feed)[(e)]	9	6.7	8.1
Cattle concentrate (complementary feed)	8	6.2	8.5
Dairy cows TMR (based on corn silage)[(e)]	15	6.3	5.5
Dairy cows TMR (based on grass silage)[(e)]	15	7.1	6.5
Dairy concentrate (complementary feed)	13	8.9	10.2
Dairy cows mineral feed (min. 40% crude ash)	8	19.5	19.5
Goats (complete feed)[(e)]	7	12.5	13.4
Sheep (complete feed)[(e)]	14	7.1	8.8
Horse (complete feed)[(e)]	6	11.9	9.9
Rabbit, breeder (complete feed)	8	10.0	12.8
Rabbit, grower/finisher (complete feed)	14	14.1	12.2
Salmon feed (wet)[(e)]	4	4.9	4.6
Salmon feed (dry)	6	7.4	7.4
Trout feed (dry)	12	10.0	11.7
Dog food (dry)	12	4.7	9.9
Cat food (dry)	16	7.4	11.4

(a): CVB, 2009. Feed Tables. Productschap Diervoeding, the Netherlands.
(b): INRA, 2004. Tables of composition and nutritional value of feed materials. Wageningen Academic Publishers, the Netherlands & INRA, Paris, France.
(c): For mineral sources element concentrations were used from Batal and Dale. 2008. Feedstuffs September 10, p. 16.
(d): For feed materials without Cu content in the INRA tables, CVB values were used to complete the data set.
(e): On DM basis.

Annex D – Copper concentration in food of animal origin

Table D.1: Copper content of different foodstuffs extracted from the German Food Composition tables

Food of animal origin	Average (range) (mg/kg fresh matter)	Food of animal origin	Average (range) (mg/kg fresh matter)
Milk		**Meat/muscle**	
Human	0.35 (0.22–0.77)	Beef, only muscle	0.87 (0.70–1.20)
Cow (raw milk)	0.10 (0.02–0.30)	Veal, only muscle	1.60 (0.90–2.40)
Goat	0.11 (0.08–0.75)	Sheep, only muscle	0.90 (0.50–1.30)
Sheep (ewe's milk)	0.15 (0.09–0.88)	Lamb, only muscle	1.70 (1.30–2.40)
Eggs		Pork, muscles only	0.88 (0.60–0.90)
Laying hens (whole egg)	0.65	Rabbit, meat	1.50
Some Fish		Chicken for fattening	0.42
Flounder	0.47	Turkey for fattening	1.10 (0.40–1.80)
Halibut	0.41 (0.26–2.30)	**Liver**	
Herring (Atlantic)	1.23 (0.75–4.40)	Beef	32
Cod	0.53 (0.15–4.70)	Calf	55 (35–79)
Mackerel	1.14 (0.55–2.00)	Sheep	76 (45–110)
Horse mackerel	0.57 (0.44–1.90)	Pig	13 (9–16)
Sardine	1.70	Chicken for fattening	3.20 (1.50–4.10)
Plaice	0.42 (0.10–5.50)	**Heart**	
Alaska pollack	0.35	Beef	3.00
Tuna	0.51	Calf	3.20 (2.90–3.40)
Eel	0.87 (0.40–0.91)	Sheep	4.50
Trout	1.47 (0.39–1.70)	Pig	4.10
Carp	0.87	**Lung**	
Salmon	1.29 (0.58–2.00)	Beef	2.60
Blood		**Kidney**	
Beef	0.90	Beef	4.30 (3.70–4.40)
		Calf	3.70
		Sheep	3.50
		Pig	7.80 (6.0–7.9)

Reference

Souci SW, Fachmann W and Kraut H, 2008. Food Composition and Nutrition Tables. Seventh Edition. MedPharm Scientific Publ. and Taylor Francis, pp. 231–580.

Tables D.2 (from D.2 (I) to D.2 (XII)). Copper content in animal tissues and products extracted from literature sources

Table D.2 (I): Published data on copper concentrations in liver, kidney and muscle in cattle. Average concentrations (mg/kg fresh weight) are given. The numbers of samples analysed are in parentheses (from López-Alonso et al., 2000)

Liver	Kidney	Muscle	Country	Reference
64.6 (437)	4.91 (427)	0.677 (438)	Spain (calves)	The study of reference
60.3 (56)	3.67 (56)	1.26 (56)	Spain (cows)	The study of reference
33.8 (180)	4.9 (178)	1.9 (181)	Australia	Kramer et al. (1983)
23.5 (1,101)	4.36 (1,226)	1.33 (1,795)	Australia	Langlands et al. (1987)
30.3 (11)	3.4 (11)	0.74 (10)	Burundi[(a)]	Benemariya et al. (1993)
56.80 (2,138)	5.00 (2,138)	–	Canada[(b)]	Salisbury et al. (1991)
137 (210)	6.69 (209)	–	Canada[(c)]	Salisbury et al. (1991)
29 (147)	5.6 (147)	1.2 (147)	Poland	Falandysz (1993)

Liver	Kidney	Muscle	Country	Reference
39 (7)	3.7 (6)	0.87 (7)	Sweden[(d)]	Jorhem et al. (1989)
28.497 (6)	4.180 (6)	3.292 (6)	Slovac Republic[(e)]	Kottferová and Koréneková (1995)
–	3.7 (70)	–	Netherlands[(f)]	Ellen et al. (1989)
65.5 (13)	4.39 (13)	–	United Kingdom[(g)]	Ministry of Agriculture, Fisheries and Food (1988)

(a): Cows.
(b): Cattle.
(c): Veal.
(d): 2 or more years.
(e): Dairy cows, heifers, bulls.
(f): 1–8 years old (mean 3.4).
(g): Cow and ox.

Reference

López Alonso M, Benedito JL, Miranda M, Castillo C, Hernandez J and Shore RF, 2000. Toxic and trace elements in liver, kidney and meat from cattle slaughtered in Galicia (NW Spain). Food Additives and Contaminants, 17, 447–457.

Table D.2 (II): Copper content in liver of ruminants (mg/kg FW) (data from feeding studies)

Species	Cu content in diet	Cu content in liver	Reference
Sheep	21.2 mg Cu/kg DM	57.5–81.3	Pal et al. (2010)
Bovine (cattle for fattening)	8.2–20.2 mg/kg TMR	2.0–18.7	Hansen et al. (2008)
Bovine (dairy cows)	11.0–17.3 mg Cu/kg TMR	37.0–157.8	Du et al. (1996), Hackbart et al. (2010)

References

Du Z, Hemken RW and Harmon RJ, 1996. Copper metabolism of Holstein and Jersey cows and heifers fed diets high in cupric sulfate or copper proteinate. Journal of Dairy Science, 79, 1873–1880.
Hackbart K, Rerreira R, Dietsche A, Socha M, Shaver R, Wiltbank M and Fricke PP, 2010. Effect of dietary organic zinc, manganese, copper and cobalt supplementation on milk production, follicular growth, embryo quality and tissue mineral concentrations in dairy cows. Journal of Animal Science, 88, 3856–3870.
Hansen S, Schlegel P, Legleiter R, Lloyd K and Spears J, 2008. Bioavailability of copper from copper glycinate in steers fed high dietary sulfur and molybdenum. Journal of Animal Science, 86, 173–179.
Pal D, Gowda N, Prasad C, Amarnath R, Bharadwaj U, Suresh Babu G and Sampath K, 2010. Effect of copper- and zinc-methionine supplementation on bioavailability, mineral status and tissue concentrations of copper and zinc in ewes. Journal of Trace Elements in Medicine and Biology, 24, 89–94.

Table D.2 (III): Copper content in bovine liver (mg/kg FW) (data from miscellaneous studies)

Type of study/variables tested	Cu content in liver	Reference
Survey (2000–2009) of EU data	20–90	Dermauw et al. (2014)
Soil fertilised with pig manure		López-Alonso et al. (2000)
Soil with 5–10 mg Cu/kg	2.9–301 (median: 39.3); n = 213	
Soil with 150 mg Cu/kg	91.2–159 (median: 112); n = 3	
Survey (data from 2005) from the Belgian Federal Food Safety Agency		Waegeneers et al. (2009)
Cu-polluted soil	3.1–279; n = 53	
Non-polluted soil	1.9–395; n = 97	

References

Dermauw V, Lopez Alonso M, Duchateau Luc, Du Laing G, Tolosa T, Dierenfeld E, Clauss M and Janssens GPJ, 2014. Trace Element Distribution in Selected Edible Tissues of Zebu (*Bos indicus*) Cattle Slaughtered at Jimma, SW Ethiopia. PLOS One 9, e85300.
López-Alonso M, Benedito L, Castillo C, Hernandez J and Shore R, 2000. The effect of pig farming on copper and zinc accumulation in cattle in Galicia (North-Western Spain). The Veterinary Journal, 160, 259–266.
Waegeneers N, Pizzolon J-C, Hoenig M and De Temmerman L, 2009. Accumulation of trace elements in cattle from rural and industrial areas in Belgium. Food Additives and Contaminants, 26, 326–332.

Table D.2 (IV): Published data on copper concentrations in pig liver, kidney and muscle (from López-Alonso et al., 2007)

Liver	Kidney	Muscle	Country	Reference
12.0 (662)	7.3 (607)	–	Canada	Korsrud et al. (1985)
12.2 (2,062)	7.58 (2,061)	–	Canada	Salisbury et al. (1991)
16 (4)	6.0 (4)	0.87 (20)	Finland	Nuurtamo et al. (1980)
–	–	0.5	Italy	Lombardi-Boccia et al. (2005)
–	7.9 (71)	–	Netherlands(a)	Ellen et al. (1989)
6.2 (804)	6.2 (805)	0.7 (926)	Poland	Falandysz and Lorenc-Biala (1989)
8.5 (661)	8.4 (663)	1.1 (658)	Poland	Falandysz (1993a)
9.0 (126)	6.1 (75)	0.9 (126)	Sweden(b)	Jorhem et al. (1989)
60.5 (13)	3.57 (13)	–	UK	MAFF (1998)
11.1 (326)	6.65 (321)	1.16 (326)	USA(c)	Coleman et al. (1992)
18.3 (282)	6.73 (281)	0.93 (281)	USA(d)	Coleman et al. (1992)

Values shown are average concentrations (mg/kg fresh weight). The numbers of samples analysed are in parentheses.
(a): Age of 4–24 months (mean = 7 months).
(b): Age of around 6 months.
(c): Market hogs.
(d): Adults.

Reference

López-Alonso M, Miranda M, Castillo C, Hernández J, García-Vaquero M and Benedito JL, 2007. Toxic and essential metals in liver, kidney and muscle of pigs at slaughter in Galicia, north-west Spain. Food Additives and Contaminants 24, 943–954.

Table D.2 (V): Copper concentrations in horse liver (mg/kg FW)

Cu content	Reference
6.9	Coenen (2013)
5.5–8.3	Elinder et al. (1981)
5.3–6.7	Koizumi et al. (1989)

References

Coenen M, 2013. Macro and trace elements in equine nutrition; in: Equine and Appled Clinical Nutrition, Eds. R. Geor, P. Harris, M. Coenen, Saunders/Elsevier, London, pp 190–228.
Elinder C-G, Nordberg M, Palm B and Piscator M, 1981. Cadmium, zinc and copper in horse liver and in horse liver metallothionin: comparisons with kidney cortex. Environmental research, 26, 22–32.
Koizumi N, Inoue Y, Ninomiya R, Fujita D and Tsukamoto T, 1989. Relationship of cadmium accumulation to zinc and copper concentration in horse liver and kidney. Environmental Research, 49, 104–114.

Table D.2 (VI): Copper concentrations in milk (mg/L; range or average with SD) extracted from feeding studies

Copper in milk	Concentration of Cu in diet (mg/kg DM)	Reference
0.17–0.20	33–36	Sol-Morales et al. (2000)
0.13–0.20	8	
0.15–0.20	15–42	Schwarz and Kirchgessner (1978)
0.036 ± 0.014	19	Pechová et al. (2008)
0.050–0.056	14.2	Rey-Crespo et al. (2014)

References

Pechová A, Pavlata L, Dvořák R and Lokajová E, 2008. Contents of Zn, Cu, Mn and Se in milk in relation to their concentrations in blood, milk yield and stage of lactation in dairy cattle. Acta Veterinaria Brno, 77, 523–531.
Rey Crespo F, López–Alonso M and Miranda M, 2014. The use of seaweed from the Galician coast as a mineral supplement in organic dairy cattle. Animal, 8, 580–586.

Schwarz FJ and Kirchgessner M, 1978. Copper and zinc contents in milk and plasma of cows after high nutritional copper supplements. Zeitschrift fur Lebensmittel-untersuchugn und- Forschung, 166, 5–8.
Sol Morales M, Palmquist DL and Weiss WP, 2000. Milk fat composition of Holstein and Jersey cows with control or depleted copper status and fed whole soybeans or tallow. Journal of Dairy Science, 9, 2112–2119.

Table D.2 (VII): Copper concentrations in milk (mg/L) from organic farms or conventional (at farm and supermarket level) production

Source	Breed	Average (range)(1)	n	Reference
Organic (farm)		0.041[a] (0.024–0.062)	22	Rey-Crespo et al. (2013)
Convent. (farm)		0.051[b] (0.038–0.065)	10	
Convent. (supermarket)		0.069[b] (0.055–0.11)	5(2)	
Organic (farm)	Holstein	0.044		Hermansen et al. (2005)
	Jersey	0.067		
Conventional (farm)	Holstein	0.053		
	Jersey	0.068		

(1): (Only for Rey-Crespo et al., 2013): Different superscript letters indicate statistically significant differences between groups.
(2): Note from the paper: 'On suspicion that the conventional milk samples were not representative of the whole population, 5 retail-supermarket milk samples (1 L of whole UHT milk) from the main conventional milk brands in the region were also analysed'.

References

Hermansen JE, Badsberg JH, Kristensen T and Gundersen V, 2005. Major and trace elements in organically or conventionally produced milk. Journal of Dairy Research, 72, 362–368.
Rey-Crespo F, Miranda M and López-Alonso M, 2013. 'Essential trace and toxic element concentrations in organic and conventional milk in NW Spain'. Food and Chemical Toxicology, 55, 513–518.

Table D.2 (VIII): Copper values per 100 g of fresh dairy products (mean ± SD) (from Reykdal et al., 2011)

Product	N	Cu (mg/100 g)
Whole milk, summer	4	0.0041 ± 0.0005
Whole milk, winter	4	0.0042 ± 0.0006
Whole milk for skyr	3	0.0046 ± 0.001
Cream	3	0.0043 ± 0.0009
Skimmed milk	3	0.0060 ± 0.0001
Skyr, industrial	3	0.0157 ± 0.0031
Skyr whey, industrial	3	0.0010 ± 0.0003
Skyr, traditional	3	0.0158 ± 0.0032
Skyr whey, traditional	3	0.0009 ± 0.0002
Milk for cheese	3	0.0036 ± 0.0003
Cheese, Gouda, second day	3	0.0261 ± 0.0055
Cheese, Gouda, ripe	3	0.0266 ± 0.0080
Cheese, whey	3	0.0016 ± 0.0003
Cheese spread from whey	2	0.0122

N: number of samples.

Reference

Reykdal O, Rabieh S, Steingrimsdottir L and Gunnlaugsdottir H, 2011. Minerals and trace elements in Icelandic dairy products and meat. Journal of Food Composition and Analysis, 24, 980–986.

Table D.2 (IX): Copper concentration in yolk and albumin of eggs from three different husbandry systems (from Giannenas et al., 2009)

Egg yolk (ng/g)			Egg albumin (ng/g)		
Conventional	**Organic**	**Courtyard**	**Conventional**	**Organic**	**Courtyard**
1,357 ± 111	1,233 ± 104	1,282 ± 108	212 ± 24	189 ± 28	254 ± 34

Values are means ± SEM.

Reference

Giannenas I, Nisianakis P, Gavrill A, Kontopidis G and Kyriazakis I, 2009. Trace mineral content of conventional, organic and courtyard eggs analysed by inductively coupled plasma mass spectrometry (ICP-MS). Food Chemistry, 114, 706–711.

Table D.2 (X): The effect of the rearing system on the ash level of the edible part of the egg and copper concentration (DW) (from Kucukyilmaz et al., 2012)

Minerals (ppm)	Organic	Conventional	Standard error mean	p value
Cu (ppm)	11.6	12.8	1.87	0.6467

Reference

Kucukyilmaz K, Bozkurt M, Yamaner C, Cinar M and Konak R, 2012. Effect of an organic and conventional rearing system on the mineral content of hen eggs. Food Chemistry, 132, 989–992.

Table D.2 (XI): Copper concentrations (mg/kg DW) in muscle tissue of farmed fish and crustaceans

Common name	Latin name	Mean mg/kg DW	Variation mg/kg DW	Reference
Fish				
Atlantic salmon	*Salmo salar*	0.3[(a)]	< 0.3–0.6[(a)]	NIFES (www)
		0.36	± 0.020 (SEM)	Johnston et al. (2006)
Seabass	*Dicentrarchus labrax*	3.89[(a)]	± 0.55[(a)] (SD)	Alasalvar et al. (2002)
		0.3	± 0.02 (SD)	Yildiz (2008)
		0.29	± 0.05 (SD)	Fuentes et al. (2010)
Sea bream	*Sparus aurata*	0.3	± 0.03 (SD)	Yildiz (2008)
Haddock	*Melanogrammus aeglefinus*	3.84	± 0.32 (SD)	Roy and Lall (2006)
Yellow perch	*Perca flavescens*	0.45	± 0.18 (SD)	González et al. (2006)
Atlantic cod	*Gadus morhua*	< 0.3[(a)]		NIFES (www)
Yellowtail	*Seriola lalandi*	2.4[(b)]	± 1.47[(b)] (SD)	O'Neill et al. (2015)
		0.6	± 0.1[(a)] (SD)	Canepa et al. (2016)
Crustaceans				
White shrimp	*Litopenaeus vannamei*	24.3	12.8–42.5	Wu and Yang (2011)

(a): Wet weight.
(b): Defatted samples.

References

Alasalvar C, Taylor KDA, Zubcov E, Shahidi F and Alexis M, 2002. Differentiation of cultured and wild sea bass (Dicentrarchus labrax): Total lipid content, fatty acid and trace mineral composition. Food Chemistry, 79, 145–150.
Canepa MM, Bubner EJ, Thomas M, Carragher J and D'Antignana T, 2016. Nutritional parameters of dorsal and ventral fillets in summer and winter from farmed yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquaculture Research, 47, 1560–1568.
Fuentes A, Fernández-Segovia I, Serra JA and Barat JM, 2010. Comparison of wild and cultured sea bass (*Dicentrarchus labrax*) quality. Food Chemistry, 119, 1514–1518.
González S, Flick GJ, O'Keefe SF, Duncan SE, McLean E and Craig SR, 2006. Composition of farmed and wild yellow perch (*Perca flavescens*). Journal of Food Composition and Analysis, 19, 720–726.
Johnston IA, Li X, Vieira VLA, Nickell D, Dingwall A, Alderson R, Campbell P and Bickerdike R, 2006. Muscle and flesh quality traits in wild and farmed Atlantic salmon. Aquaculture, 256, 323–336.
O'Neill B, Le Roux A and Hoffman LC, 2015. Comparative study of the nutritional composition of wild versus farmed yellowtail (*Seriola lalandi*). Aquaculture, 448, 169–175.

Roy PK and Lall SP, 2006. Mineral nutrition of haddock (*Melanogrammus aeglefinus* L.): A comparison of wild and cultured stock. Journal of Fish Biology, 68, 1460–1472.
Wu X-Y and Yang Y-F, 2011. Heavy metal (Pb, Co, Cd, Cr, Cu, Fe, Mn and Zn) concentrations in harvest-size white shrimp *Litopenaeus vannamei* tissues from aquaculture and wild source. Journal of Food Composition and Analysis, 24, 62–65.
Yildiz M, 2008. Mineral composition of fillets of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurate*). A comparison of cultured and wild fish. Journal of Applied Ichtyology, 24, 589–584.

Table D.2 (XII): Copper contents in animal tissues and products (mg Cu/kg fresh matter) from feed supplementation studies. Data from Van Paemel et al., 2010;(c) unless otherwise specified (taken from EFSA FEEDAP Panel, 2012)(d)

Species/category	Duration	Dietary Cu (mg Cu/kg feed)	Liver	Muscle	Egg
Pig	161 days	15	7.9	0.44	
		30	13.4	0.43	
Pig(e)	Not specified	22	8.8	1.5	
	Not specified	35	16.8	0.7	
	Not specified	14.4/10.2(a)	22.4	0.98	
	Not specified	15/17(a)	36.4	1.08	
Laying hens	28 days	27	13.4		1.04
	28 days	23.8			< 0.9 yolk/< 0.3 white
		30.8			< 0.9 yolk/< 0.3 white
	56 days	8.1	5.0		1.61 yolk/0.22 white
		29.9	5.4		2.02 yolk/0.23 white
Chicks	21 days	9.8	7.7		
Steers	177 days	0(b)	25.3	0.87	
		10(b)	113	1.1	
		20(b)	152	0.75	
Dairy cows	60 days	5.5	67.5		
		43	191.5		
Sheep(e)	Not specified	3.5	120	1.25	
Goat(e)	Not specified	4	40	1	
	Not specified	19	140	2	
	Not specified	6	126	0.45	
Rainbow trout	28 days	11.4	38.4	0.26	
		277.8	45.2	0.32	
Atlantic cod(f)	2 years	2.8		0.4	
		10.1		2.7	
Sea Bass (Farmed)(g)	Not specified	11.3		1.1	
		14.8		0.99	

(a): Copper concentration in Grower/Finisher.
(b): Supplemented copper. Background in feed not reported.
(c): Van Paemel M, Dierick N, Janssens G, Fievez V and De Smet S, online, 2010. Selected trace and ultratrace elements: biological role, content in feed and requirements in animal nutrition – Elements for risk assessment. Technical Report submitted to EFSA. http://www.efsa.europa.eu/en/supporting/doc/68e.pdf
(d): EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Scientific Opinion on the safety and efficacy of copper compounds (E4) as feed additives for all animal species: cupric sulphate pentahydrate based on a dossier submitted by Manica S.p.A. EFSA Journal 2012;10(12):2969, 38 pp. doi:10.2903/j.efsa.2012.2969
(e): European Commission, 2003a. Scientific Committee on Animal Nutrition (SCAN) delivered report on the use of copper in feedingstuffs (19 February 2003). Available online: http://ec.europa.eu/food/fs/sc/scan/out115_en.pdf
(f): Herland H, Cooper M, Esaiassen M and Olsen RL, 2011. Effects of Dietary Mineral Supplementation on Quality of Fresh and Salt-Cured Fillets from Farmed Atlantic Cod, *Gadus morhua*. Journal of the World Aquaculture Society, 42, 261–267.
(g): Trocino A, Xiccato G, Majolini D, Tazzoli M, Tulli F, Tibaldi E, Messina CM and Santulli A, 2012. Levels of dioxin-like polychlorinated biphenyls (DL-PCBs) and metals in European sea bass from farms in Italy. Food Chemistry, 134, 333–338.

Annex E – Copper concentration in food of animal origin. Monitoring of veterinary medicinal product residues and other substances in live animals and animal products, year 2014. Details of non-compliant samples on copper

Species/ group	Country	Matrix analysed	Number of samples analysed	Number of NC[a] results	% of NC results	Concentration range (mg Cu/kg matrix) of NC samples
Bovine	CZ	Liver	34	15	44.1	71.9–227
	DE	Liver	275	168	61.1	30.6–356
Pigs	CZ	Liver	37	1	2.7	77.6
	DE	Liver	1,348	149	11.1	31.0–343[b]
Sheep/goats	DE	Liver	16	11	68.8	36.9–278
		Kidney		1	6.3	67.0
Poultry	DE	Liver	131	2	1.5	67.0–82.0
Farmed game	DE	Liver	14	3	21.4	34.2–132
Wild game	DE	Liver	75	5	6.7	6.6–158

(a): NC: non-compliant.
(b): A value of 653 mg Cu/kg was considered outlier and not included.