

Ch 5: Micronutrients - Part II: F, Al, Mo and Co

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Abstract

Ultratrace element is a relatively new term defined as those elements with an established, estimated, or suspected dietary requirement of minute amount, generally of the order of $\mu\text{g/day}$. This chapter focuses on fluorine (F), aluminium (Al), molybdenum (Mo) and cobalt (Co). Whilst diet is the principal source of Al, Mo and Co found in the body, inadvertent ingestion of dental hygiene products accounts for a significant proportion of F intake. Apart from fluorine, the influence of other ultratrace elements on oral health, and in particular dental caries, has not been fully established. The calcified tissues contain 99% of body F. During tooth development, ingested (systemic) F is incorporated into the apatite crystals of the developing tooth which helps in improving resistance to acid demineralisation. However, the presence of low but constant levels of topical F in the fluid phase at the tooth enamel surface are more important in controlling tooth decay for people of all ages. An adequate intake (AI), from all dietary and non-dietary sources, is estimated as 0.05 mg/kg body weight/day for children older than 6 months and adults, based on estimated intakes that have been shown to reduce the incidence of dental caries while minimising adverse health effects such as dental fluorosis. An inverse relationship between incidence of dental caries and levels of Al in drinking water, food and soils has been indicated by some epidemiological studies. Co and Mo, whilst occasionally showing potential beneficial oral health effects in laboratory experiments, do so at concentrations much higher than found in vivo.

Introduction

Ultratrace element is a relatively new term defined as those elements with an established, estimated, or suspected dietary requirement of minute amount, generally of the order of $\mu\text{g/day}$ [1]. These elements may enter the body through absorption from water and diet as well as inhalation from the atmosphere.

Apart from fluorine, the influence of other ultratrace elements on oral health, and in particular dental caries, has not been fully established. Teeth, particularly deciduous teeth, have been used as a biomarker of exposure to elements and therefore examined for their elemental composition by many.

Tooth enamel contains both organic and inorganic phases. The inorganic phase is composed of calcium-deficient calcium hydroxyapatite (HA) with small amounts of incorporated trace and ultratrace elements. The enamel hardness and optical properties can be affected by the organisation and size of the apatite crystals, which in turn can be influenced by the presence of impurity elements [2].

The mineralisation of primary teeth commences prenatally between 14- and 16-week gestation and settles postnatally at 1.5 to 3 months for incisors, 9 months for canines, and 5.5 to 11 months for molars [3]. It has been demonstrated that elements in circulation are primarily deposited into the structure of the tooth during calcification, before eruption. At the post-eruptive stage, elements might be incorporated into or lost from the enamel through exchange with either the blood supply to the pulp cavity via the dentine or with saliva, and likewise the dentine with the pulp [4].

Although the list of ultratrace elements comprises 14 elements (see chapter 1), this chapter will concentrate on fluorine, aluminium, molybdenum and cobalt, with a special focus on fluorine due to its well-established (as fluoride anion) cariostatic role. We note that some metal ions, whilst occasionally showing potential beneficial effects in laboratory experiments, almost always do so at concentrations much higher than found in vivo and that may be too high for exploitation (for reasons of e.g. toxicity, formulation incompatibility, etc).

Fluoride

Fluorine is a natural element found at different concentrations in water, air, soil and food [5]. Since fluorine is extremely electronegative, it mainly appears as fluoride compounds rather than in its free elemental form. There are two general forms of fluoride (F); organic and inorganic [6, 7]. The organic fraction refers to F which is covalently bound to carbon and therefore is 'non-diffusible', 'non-absorbable' and 'non-adsorbable' on calcium hydroxyapatite; whereas the inorganic fraction denotes 'ionic' and 'ionisable' F which is 'diffusible', 'absorbable' and 'adsorbable'. The ionic form is represented by free F ions in solution and the ionisable form is bound to (i) protons as in HF at low pH, (ii) metal ions such as Ca^{2+} , Mg^{2+} , Al^{3+} etc, (iii) mineral/organic sediments in saliva and organic molecules like proteins in plasma, and inorganic F incorporated into the apatite lattice of bones and

teeth [6]. The inorganic form of F is the one of importance in dentistry, medicine and public health [7].

Exposure to F and its sources

The contribution of air-borne F to total daily F exposure is <0.1% for an adult and $\leq 0.3\%$ for a child living in non-industrial areas [8]. Since F is the 13th most copious element in the earth's crust, F in soil could be a source of inadvertent exposure, primarily for children [8]. However, diet and inadvertent ingestion of dental care products containing F are the main sources of systemic F exposure (Figure 1).

Dentifrice ingestion can be a considerable source of systemic F intake in children younger than 6 years of age, as they are not in full control of their swallowing reflex [18]. Most toothpastes usually contain 1000-1450 mg F/kg and therefore one gram of toothpaste contains about 1.0-1.5 mg F.

Depending on the age of the child, the amount of dentifrice used and rinsing habits, children could ingest 0.13 to 0.59 mg F per tooth brushing session [12-14, 19, 20].

F intake studies of infants have indicated diet as the sole source of F intake for 87% of infants aged 1–12 months [9]. In young children, the contribution of diet to total F intake could be up to 70% [10-13].

Dietary sources of systemic F intake include drinking water, foods and drinks prepared with water or which contain F naturally, as well as dietary F supplements such as artificially fluoridated water, milk and salt. After water, tea could potentially be the most significant source of dietary F intake, particularly in countries where tea drinking is common. The range of F content of tea leaves and infusions is very wide: from 26-820 mg/kg and 0.29 to 8.85 mg/l [14, 15], respectively. Fresh fruit juices and carbonated soft drinks are low in fluoride, ranging from 0.009 to 0.931 mg/l [14, 16].

Generally, the F content of beverages depends on the F concentration of the water used for processing and preparation. The F level of soy milks could be up to 0.96 mg/l [17]; whereas F levels are less than 0.05 mg/l in human breast milk and cow's milk [14, 18, 19]. The F content of powdered infant milk formulas (IMF) ranges from 0.01 to 3.71 mg/kg. When reconstituted with fluoridated water (typically containing 1 mg F/l), the F concentration of IMF ranges from 0.49 to 1.53 mg/l [20, 21]. Fluoride levels of ready-to-feed infant milks are significantly lower, up to 0.030 mg/l [22]. The levels of F in raw fruits and vegetables as well as in unprocessed foods and beverages are low. Normally, the F

content of meat is low, but levels in fish and shellfish with bone and skin could be over 10 mg/kg [14, 23].

The main contributory sources to dietary F vary between fluoridated and non-fluoridated areas. The consumption of all drinks, including water, could provide 59% and 32% of a child's dietary F intake in optimally- and non-fluoridated areas, respectively [24].

Estimated safe and adequate F intake

Inadequate information is currently available to set any Dietary Reference Value (DRV) for F. However, an adequate intake (AI) was suggested by the US Institute of Medicine (IOM) [25] based on estimated intakes that have been shown to reduce the incidence of dental caries while minimising unwanted health effects. For infants younger than 6 months, the AI is set at 0.01 mg/day in line with the amount of fluoride that a breast-fed infant receives from human milk. The AI, from all dietary and non-dietary sources, is estimated as 0.05 mg/kg body weight/day for children older than 6 months and adults.

An F intake of 0.1 mg/kg body weight/day is proposed by IOM as the upper limit (UL) of F for infants and children up to 8 years old [25]. This suggested UL was based on a high degree of certainty that a chronic systemic F ingestion of less than that by children at risk of dental fluorosis was linked with a low prevalence (<10%) to the milder forms of dental fluorosis [25]. For children older than 8 years and adults, a UL of 10 mg F/day has been determined [25], based on data from F exposure studies reporting a small risk of development of preclinical or stage 1 skeletal fluorosis in individuals who had received 10 mg F/day for more than 10 years.

Metabolism of F

Some systemic absorption of F from dental products with acidic formulations, such as APF (acidulated phosphate fluoride) gels and SnF₂ solutions, may occur in the oral cavity, even when they are not swallowed [7]. Although the absorption of F commences in the oral cavity, the major sites for absorption of F are the stomach and proximal small intestine where approximately 80-90% of orally-ingested F is absorbed by passive diffusion [7]. F is one of the few nutrients that is absorbed from the stomach, with gastric absorption accounting for 20-25% of total F absorption. The extent of F absorption from the stomach is inversely related to the pH of the stomach contents and the rate of

gastric emptying [26]. The remaining ingested F, which is not absorbed in the stomach (i.e. 70-75%), is then absorbed from the upper small intestine which has got a huge capacity for F [26]. Due to rapid absorption from the gastrointestinal tract, the levels of F in plasma increase rapidly after ingestion, reaching a peak within 20-60 minutes, depending on the time, amount and type of food ingested beforehand [27, 28]. F is fairly rapidly distributed from plasma to the soft tissues and organs in the body, which contain < 1% of body F, and incorporated into the calcified tissues, which contain 99% of body F [28]. In areas with optimal levels of F (0.7-1.0 ppm) in water, the F concentrations of bones and dentine range from 0 to 396 $\mu\text{g/g}$ (mean 176 $\mu\text{g/g}$) and 59 to 374 $\mu\text{g/g}$ (mean 215 $\mu\text{g/g}$), respectively; whereas enamel F concentration is lower, ranging from 0 to 192 $\mu\text{g/g}$ (mean 103 $\mu\text{g/g}$) and reflects the level of F exposure during its formation [28, 29]. The F concentration of whole saliva is variable and higher than that in plasma because of topically-applied F retained in the mouth. However, compared to F concentrations in plasma, ductal saliva F concentrations are slightly lower, with the ratios of submandibular saliva-to-plasma of 0.9 and parotid saliva-to-plasma of 0.8. The F concentration of gingival crevicular fluid is marginally higher than that in plasma [28]. A variable amount of ionic F, that enters the renal tubules, is reabsorbed and returned to the systemic circulation and the remainder is excreted in urine. In children, almost 55% of absorbed F is retained in the body under normal conditions, whereas the corresponding figure is 35% in adults [30].

Since gastric absorption, distribution and renal excretion of F are pH-dependent, any changes in the acid-base equilibrium in the body could affect F metabolism. Body acid-base balance can be influenced by diet through the supply of acid and alkaline precursors from foods [31]. Protein-containing foods such as meat, cereals and dairy foods generate H^+ ions; whereas most fruit and vegetables supply base precursors as well as large amounts of Mg and K in the diet. Therefore, diet high in fruit and vegetables might lead to a rise in urinary pH, increasing renal F excretion and consequently resulting in negative F balance. The composition of diet could affect F absorption; the absorption is reduced in the presence of a significant amount of divalent or trivalent cations; whereas high levels of dietary fats may enhance F absorption.

Oral Health Effect of F

Although F only appears in trace amounts in the body, it is of nutritional and public health importance and considered as a valuable nutrient by the American Dietetic Association [32] because of its role in the mineralisation of bones and teeth. The US Food and Drug Administration (FDA) [33] has regarded F as the only substance for the prevention of dental caries. Fluoride achieves its anticaries effect through systemic and topical actions. During tooth development, ingested (systemic) F is incorporated into the apatite crystals of the developing tooth which helps in improving resistance to acid demineralisation. In addition, the ingested F can return to the oral cavity through saliva and crevicular fluid and influence de- and re-mineralisation processes. However, it has long been believed that the presence of low but elevated levels of topical F, which are independent of systemic F, in the fluid phase at the tooth enamel surface are more important in controlling tooth decay for people of all age groups [34-36]. The so-called demineralisation – remineralisation balance of the tooth benefits from F in several ways [37, 38]. Fluoride helps lessen demineralisation of tooth enamel and dentine hypersensitivity not only by reducing acid solubility of enamel but, more importantly, by encouraging the uptake of tooth minerals (calcium and phosphate) and the precipitation of fluoridated hydroxyapatite within the enamel, and consequently by reducing the net rate of transport of minerals out of the enamel. Fluoride also affects the cariogenic bacteria in oral plaque that produce the acid, which can dissolve teeth. Fluoride enters bacterial cells and is able to interfere with their acid production and, consequently, decreases the potential for enamel destruction.

Chronic exposure to excessive systemic F, during critical periods of tooth development, could result in fluorosis in both primary and permanent teeth. The most important period for development of dental fluorosis in permanent incisors as well as the first permanent molars is the first 3 years of life, especially between 6 and 24 months; whereas for the later developing permanent canines and premolars the risk period could be up to 8 years of age [39, 40]. Many of the changes triggered by F are dose-related and are associated with cell/matrix interactions as the teeth are forming [41]. Mild cases of dental fluorosis are characterised by the white opaque appearance of fluorosed enamel, caused by a hypomineralised enamel subsurface. In severe cases of dental fluorosis, pitting and a loss of enamel surface occurs, leading to brown staining [41].

Excessive intake and Toxicity

Similar to most nutrients, excessive ingestion of F could have some side effects or even be toxic. Skeletal fluorosis, a chronic metabolic bone and joint disease caused by systemic ingestion of large amounts of F over many years during periods of bone modelling (growth) and/or remodelling, results in weaker bones and stiffness and pain in the joints [29].

Acute F toxicity can happen when F is ingested as a large amount in a single dose or in multiple doses within a few hours. Since the first organ to be affected by systemic acute exposure is the stomach, clinical systemic toxicity starts with gastric signs and symptoms, ranging from some degree of nausea to abdominal pain, haemorrhagic gastroenteritis, vomiting and diarrhoea [42]. Dental products are the most common sources of acute overexposure due to their rather high F concentrations and their appealing flavours [42]. The 'probably toxic dose' (PTD) of F is 5 mg/kg body weight (11 mg per kg body weight of NaF) and death can occur with a F ingestion of 16 mg/kg body weight [43].

Aluminium

Aluminium (Al) is the most abundant metal and the third most copious element; making up about 8% of the earth's crust. Al is virtually never found in the elemental form, due to its strong affinity for oxygen [44]. It is redistributed in the environment mainly by natural and industrial processes and the released Al into air is the main source of Al in salt- and freshwater.

Exposure and metabolism of Al

The primary source of Al is food, which is originated from natural sources including water, food additives, and contamination by Al utensils and containers [45]. Apart from tea, the Al content of foods is less than 5 µg/g [45]. Bioavailability of Al from water and solid food is 0.3% and 0.1-0.3%, respectively, whereas from occupational inhalation exposure, in industrial areas, is about 2% [46]. After absorption, approximately 95% of Al becomes bound to transferrin and albumin intravascularly. Al mainly accumulates in bone, brain, liver and kidney; with bone as the major site of deposition in humans. It is effectively eliminated from the body via the kidneys [47].

Al was added to the list of nutritionally essential ultratrace elements in the 1990s [1]. Al has been suggested to activate the enzyme adenylate cyclase, enhance calmodulin activity, stimulate DNA synthesis in cell cultures, and stimulate osteoblasts to form bone through activating a putative G-

protein-coupled sensing system [1]. On the other hand, a positive association between levels of Al in food and water and the risk of cognitive impairment or Alzheimer's disease has been suggested by some epidemiological studies [47]. High concentrations of Al in cells has also been linked to the alteration of the activity of several enzymes and secondary messenger pathways and the impairment of mitochondrial function, leading to oxidative stress [48].

A tolerable weekly intake (TWI) of aluminium of 1 mg/kg/week has been recommended by FAO/WHO as well as the European Food Safety Authority (EFSA) [44]. The lowest no observed adverse effect level (NOAEL) for toxicity to the developing nervous system has been suggested, by EFSA, as 10–42 mg/kg/d [44].

Oral health effect of Al

An inverse relationship between incidence of dental caries and levels of Al in drinking water, food and soils has been indicated by some epidemiological studies [49]. Determination of Al concentrations in the enamel and dentine of 314 human deciduous teeth showed a significantly higher Al concentration in sound enamel and dentine without caries than those with caries [4]. Potential cariostatic properties of topically applied Al have also been demonstrated by clinical trials [50] and animal studies [51]. Enamel dissolution experiments have indicated an increase in the resistance of hard tissue to acidic challenges after topical application of Al solutions [52-54].

The primary mechanism for the potential cariostatic action of Al has been linked to its steady incorporation at the surface of both sound and demineralised enamel [49, 54, 55], which results in the formation of tightly bound, very acid-insoluble $AlPO_4$ -type compounds within or upon the hard tissue [53]. Al can replace calcium, and perhaps phosphorus, in apatite minerals. The formation of very insoluble $Al(OH)_2H_2PO_4$ might be as a result of thermodynamically displacing three Ca atoms by two Al atoms without loss of P in the HA lattice [56].

It has been proposed that in acidic conditions producing a Ca-deficient mineral lattice environment, interaction between Al and apatite phosphate may be favoured, leading to the formation of a more stable, insoluble mineral [57]. Thus, Al may inhibit the initiation and progression of caries by forming very acid-insoluble reaction products within the surface enamel or incipient lesions [52].

Al has also been suggested to enhance both the systemic and topical effects of F [49, 58]. Application of topical F after pre-treatment of enamel with water-soluble Al solutions has been shown to increase, compared to F treatment alone, uptake, depth of penetration, and retention of F in the hard tissue in rat caries models [49]. The Al–F combination has been advocated to have a stronger inhibitory effect on acid dissolution of powdered enamel and synthetic HA than those of these substances separately [59]. However, no substantial cariostatic benefit of combined Al–F treatment protocols over implementation of F alone was found in several in vitro, animal and human studies [51, 60, 61]. Al has also been suggested to inhibit the formation of dental plaque and plaque acidogenicity [49, 50]. A significant reduction in salivary levels of oral *Streptococci* (*S. mutans*, *S. mitis* and *S. salivarius*) has been reported in children rinsing daily with a mouthwash containing hydrated aluminium potassium sulphate (alum: $KAl(SO_4)_2$) [62].

Cobalt

Cobalt naturally occurs in rocks, soils, water and vegetation in two oxidation states (Co^{2+} and Co^{3+}); although the Co^{3+} form is thermodynamically unstable under typical redox and pH conditions [63]. Since Co is an integral component of vitamin B12 complex, it has been named as an essential trace element [64]. In humans, Co is a prerequisite for production of red blood cells, effectively stimulating the production of haemoglobin and therefore preventing anaemia [44].

Exposure and metabolism of Co

The main source of Co is diet, with coffee, chocolate, fish, green leafy vegetables and cereals being the richest sources [65]. Average exposure to Co varies between 21 and 33 $\mu\text{g}/\text{d}$ for adults and 13–24 $\mu\text{g}/\text{d}$ for children [44]. No report of cobalt deficiency in normal individuals fed a balanced diet has been documented. However, it has been reported that obese children might have abnormally low levels of Co in their blood and may need supplementation [66].

Although water-soluble Co salts are mainly absorbed in the gastrointestinal tract, a significant uptake of cobalt can also occur through the lungs following inhalation [67]. It is circulated in the body via blood, bound to albumin, and gradually taken up and stored in the liver, kidney, pancreas and heart [44]. Renal excretion is the main route of Co excretion from the body which is initially rapid but

decreasing over the first days, followed by a second, slow phase lasting several weeks, and with retention in tissues for several years [67].

Guidelines on safe Co intake for humans have been established by some health authorities. A Co dosage of 1400 µg/d has been suggested by the UK Expert Group on Vitamins and Minerals as an amount that is unlikely to produce adverse health effects in adults [68], whereas the European Food Safety Authority has suggested a dose of 600 µg/d [69].

Although essential in minute amounts, Co is genotoxic and mutagenic to mammalian cells at higher concentrations. Based on experimental animal studies, it is classified as “possibly/probably carcinogenic to humans”. In cultured human cells, Co has been shown to act as a topoisomerase II poison; encouraging DNA strand breaks and inducing the formation of covalent protein-DNA complexes [70]. High doses of Co have also been suggested to cause aneuploidy (abnormal number of chromosomes). Haematological and endocrine toxicity, including polycythaemia and deficits in iodine uptake have also been associated with high doses of Co [71].

Oral health effects of Co

Investigations of the potential effects of Co on dental conditions are limited. In a study by Dreizen et al [72], whole saliva from 37 adults was analysed for Co by a colorimetric method. They found detectable amounts of Co in only 27% of samples with a wide range of concentration from 0.04 to 0.12 µg/ml. When the latter amount was added to whole saliva, Co failed to influence the growth of *L. acidophilus* in vitro; whereas a substantial decrease in acid production was observed when the amount of added Co was almost 20 times more.

Elkabouss et al [73] reported that Co^{2+} replaced Ca^{2+} in synthetic hydroxyapatite, following the equation $\text{Ca}_{10-x}\text{Co}_x(\text{PO}_4)_6(\text{OH})_2$, with an exchange limit of 1.35 wt% Co. Both these and other authors [85] also observed that Co-substituted apatites had improved crystallinity, consistent with lower solubility, although the Co amounts in these in vitro model studies were hardly ‘trace’.

Ghadimi et al [2] found that Co^{2+} incorporation in the enamel of extracted teeth had a strong negative association with carbonate type B (carbonate ion substituting for phosphate), again consistent with reduced solubility. These authors appear to conclude that the Co (and other trace metals) most

probably came from dental prostheses, rather than from the diet (a possibility also mentioned elsewhere [74]).

Molybdenum (Mo)

Molybdenum (Mo) is the 54th most abundant element in the Earth's crust and the 25th most copious element in seawater at an average concentration of 100 nM. It is an important element for the survival of animals and the only metal of the 2nd transition row (4d) of the periodic table with demonstrated biological activity for humans [75]. Mo is generally found in the form of the oxyanion molybdate (MoO_4^{2-}), which is the only recognised source of Mo that can be taken up from the environment by organisms [75].

Exposure and metabolism of Mo

The main dietary sources of Mo are legumes, such as beans, lentils and peas. However, the Mo content of plants depends on the Mo content of soil and water and other environmental conditions. Fruit, vegetables and animal products are generally low in Mo [76]. The average daily Mo intake has been reported to range from 100 to 200 μg [77]. When taken in liquid form, its intestinal absorption is relatively rapid; however, the absorption rate is reduced and delayed with composite meals [77]. Mo retention in the body is regulated by urinary excretion and the retention rate of nutritive Mo intake in the body has been reported to range from 13% to 56% with an average of 36% [78]. There is no concise and clear recommendation for Mo intake. The UK Department of Health [79] has suggested a safe intake of 50-400 $\mu\text{g}/\text{day}$ for adults and 5-1.5 $\mu\text{g}/\text{kg bw}/\text{day}$ for infants and children. An intake of 15-250 $\mu\text{g}/\text{day}$ has been recommended by the US National Research Council [80] as adequate intake, whereas the US Institute of Medicine [81] has suggested an RDA of 45 $\mu\text{g}/\text{day}$.

The main acknowledged function of Mo in humans is to act as a catalyst for enzymes such as nitrogenase, nitrate reductase (NR) and xanthine oxidase (XO), which are crucial for human health. In nature, more than 50 different Mo-dependent enzymes are known; most of them of bacterial origin with only four known mammalian. All Mo-enzymes, with the exception of nitrogenase, bind Mo through a pterin-based prosthetic group forming the so-called molybdenum cofactor (Moco) [75].

In humans, Mo toxicity is extremely rare and limited to areas with high amounts of Mo in water or soil. Cases of Mo toxicity were reported in Armenia where dietary Mo intake was 10-15 mg/day. The symptoms of Mo toxicity included aching joints and an increased incidence of a gout-like syndrome, with elevated blood levels of Mo, uric acid, and xanthine oxidase [82]. A UL of 2 mg/day has been suggested based on an animal study displaying adverse reproductive effects in female rats [82].

Oral health effect of Mo

The possible role of Mo on dental caries has been explored, mainly in 1960s and 1970s, through animal experiments and epidemiological studies. Most of the epidemiological studies suggest an anticaries effect of Mo on teeth [83-87]; whereas the evidence from animal experiments is less clear. A definitive anticaries mode-of-action for Mo is not known. Mo could affect enamel solubility, production of bacterial acid and/or morphology of the teeth [88].

Mo can be incorporated into enamel at both pre- and post-eruptive stages and might therefore affect the physical-chemical characteristics of the enamel [88]. However, concentrations are low and unlikely to influence enamel or dentine solubility. A median Mo concentration of 2 µg/g, with a wide range from 0 to 39 µg/g, has been reported in enamel of human permanent teeth [89]; whereas the Mo concentration in dentine is considerably lower (0.26 µg/g) [90].

Likewise, in vitro experiments suggest that Mo concentrations well in excess of those found in vivo are required to affect plaque acid production [8]. It has been observed that Mo may enhance the effect of fluoride in inhibiting enamel demineralisation [12] or promoting remineralisation [13] in simple in vitro studies. However, even here, the Mo concentrations required were arguably unrealistically high.

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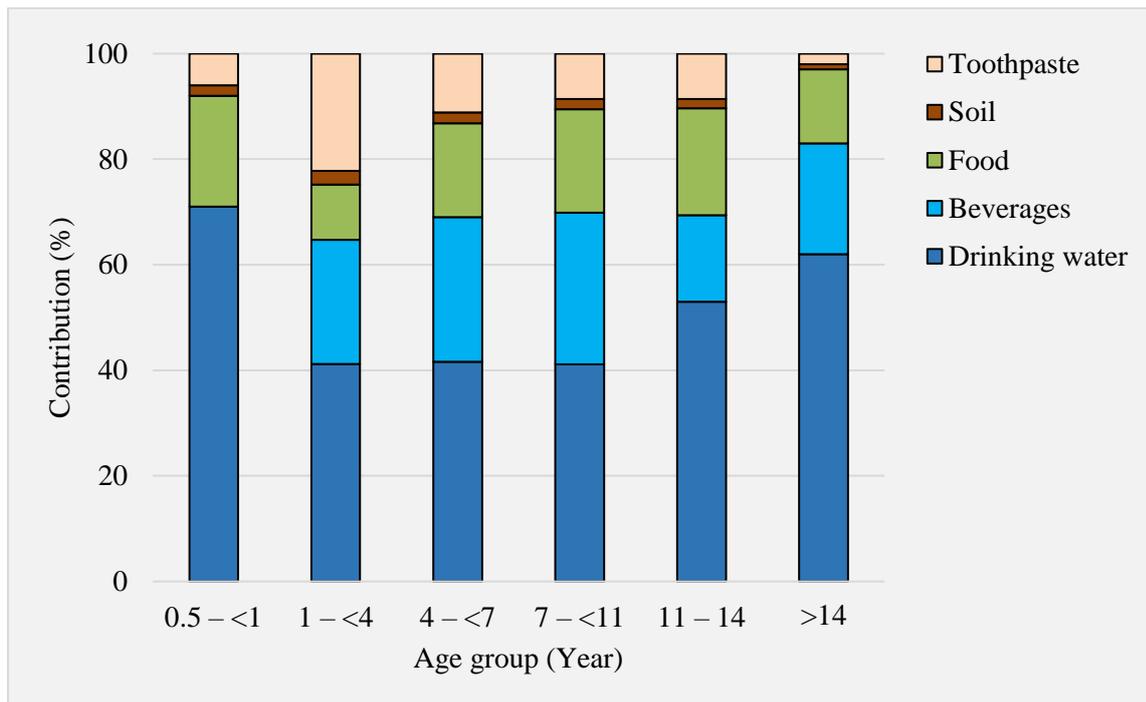


Figure 1. Contribution of diet (food and beverages), unintentional toothpaste ingestion and soil to total daily fluoride intake for individuals receiving tap water containing 0.87 mg/l fluoride. Adapted from Table 7-1 of ref. [8].