Potential dietary, non-metabolic accumulation of arsenic (As) in seaweed-eating sheep's teeth: Implications for archaeological studies

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Abstract

Evaluating the extent of an individual’s exposure to arsenic, (potentially) indicative of proximity to smelting activities, poisoning, or dietary history, has proven difficult in archaeological contexts due to uncertainties surrounding how arsenic biogenically accumulates in the tissues commonly found at archaeological sites such as bone and tooth, in addition to issues of diagenesis. In this study, teeth of modern sheep naturally exposed to high amounts of arsenic by means of seaweed in their diet are compared to the teeth of a less exposed ‘control group’ of modern sheep consuming predominantly grass.

Through analysis of total arsenic and other element concentrations in samples of enamel, cementum and dentine by hydride generation atomic fluorescence spectrometry (HG-AFS), as well as by bioimaging of radial tooth sections of sheep molars by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), this research demonstrates that arsenic in the teeth of sheep exposed to dietary arsenic predominantly accumulates in the infundibulum and occlusal dentine. The major route of uptake of arsenic in these teeth is therefore likely not by ingestion and metabolisation during growth of the tooth, as is thought to be the case for lead and barium, but rather due to direct surface contact, potentially even occurring during mastication. The implications of this type of *in vivo* chemical alteration of teeth for archaeological trace element studies are explored.

Keywords:
Dentine
Animal husbandry
Environmental pollution monitoring
LA-ICP-MS bioimaging
North Ronaldsay Sheep
Lead (Pb)
Trace elements

1 Introduction

Human and animal skeletal remains are often utilised as archives of environmental and dietary exposure to trace elements, whereby the concentrations of certain elements in the sampled tissue are usually used as indicators of the degree of exposure to these elements (Budd et al., 2000; Dolphin et al., 2013; Maurer et al., 2011; Millard et al., 2014; Reynard and Balter, 2014; Stadlbauer et al., 2007; Trueman and Tuross, 2002; Vernois et al., 1988; Wright et al., 2009). A prerequisite for such research on archaeological material is an understanding of how exactly elemental concentrations in the sampled tissues are related to exposure to these elements during life, and how diagenetic changes may affect the samples (Budd et al., 2000; Farnum et al., 1995; Hedges et al., 1995; Kohn et al., 2013; Martínez-García et al., 2006, 2005; Maurer et al., 2011; Millard, 2006; Nielsen-Marsh et al., 2006).

In case of arsenic (As), the exposure-correlated accumulation of inorganic As in modern organic bodily tissues such as hair, nails, and internal organs is well documented and the study of such samples can reveal e.g. dietary histories, drinking water contamination and poisoning (Chowdhury et al., 2000; Cornelis and De Kimpe, 1994; Feldmann et al., 2000; Samanta et al., 2004). However, the case for skeletal tissues is less clear: In studies of modern human bones, elevated concentrations of As have been found in individuals exposed to airborne As due to smelting and refining processes (Lindh et al., 1980), and other industrial emission of As (Brodziak-Dopierała et al., 2011). Arguably, some older evidence also exists of elevated As concentrations in bones due to ingestion of As (Brouardel and Pouchet, 1889; Chittenden, 1885), though this may be unreliable. In contrast to this, several studies documented that As concentrations in skeletal tissues of exposed individuals were not significantly higher than those of unexposed individuals (e.g. Bocio et al., 2005; Ismail and Roberts, 1992; Jurkiewicz et al., 2004; Lindh et al., 1980; Wiechula et al., 2003; see Table A.1 in the appendix).

These latter cases may well be due to the difference between exposure levels deemed to be “elevated” and “normal” being too small to have any significant impact on the skeletal tissues of the sampled individuals, so that it is quite possible that increased exposure to As do indeed lead to measurably higher skeletal concentrations. However, as little other direct evidence of the impact of exposure to As on skeletal tissues is available, current evidence of the relationship between exposure to As and skeletal concentrations is still inconclusive. Furthermore, the relationship between means of exposure (e.g. inhalation, ingestion or skin absorption) and skeletal As concentrations has not yet been adequately characterised.

Despite this, drawing parallels between As and other metals that accumulate in skeletal tissues according to the degree of exposure, such as lead (Barbosa Jr et al., 2005), has led to the assumption that As concentrations in skeletal tissues can serve as proxies for dietary and inhalation exposure to As during life and the application of these approaches to archaeological materials. Arsenic concentrations of human and faunal skeletal remains have been determined with the aim of investigating past exposure to As (Goodwin et al., 2007; Rasmussen, 1974; Stadlbauer et al., 2007; Zhou et al., 2004) due to dietary uptake (Djingova et al., 2004; Farnum et al., 1995); contaminated drinking and irrigation water (Swift et al., 2015); and inhalation of airborne As compounds produced in metallurgical processes (Dirilgen et al., 2006; Oakberg et al., 2000; Özdemir et al., 2010). In a number of cases, the measured As concentrations were judged to be too high to be solely of biogenic origin (Farnum et al., 1995; Güner et al., 2011; Özdemir et al., 2010; Pike and Richards, 2002; Rasmussen et al., 2009), and have instead been attributed to diagenetic uptake of As.

In light of this issue, several studies of archaeological material have focussed on identifying, removing or accounting for diagenetic changes to As concentrations by including the analyses of burial soils surrounding the sampled skeletal material to evaluate the potential for and likely extent of diagenetic changes and/or using other elements as markers for diagenesis (e.g. Özdemir et al., 2010; Rasmussen et al., 2009; Shafer et al., 2008; Swift et al., 2015). The exact diagenetic processes affecting As concentrations are currently not well understood (Dudgeon et al., 2016; Pike and Richards, 2002), and neither is the form in which As resides in diagenetically altered (or even in unaltered) skeletal tissues. However, since arsenate (AsO_4^{3-}) may substitute for phosphate (PO_4^{3-}) in laboratory-synthesised samples of hydroxyapatite (e.g. Lee et al., 2009; Mahapatra et al., 1987), this has also been posited for diagenetic replacements in skeletal bioapatite (Dudgeon et al., 2016; Shafer et al., 2008).

In order to evaluate if diagenetic uptake of As may be distinguished from biogenically incorporated As, Dudgeon *et al.* studied the spatial distribution of As in archaeological bones and teeth (Dudgeon et al. 2016). Finding a different distribution pattern for As than for diagenetic "overprinting" indicator elements such as strontium, barium and uranium, they posited that this indicates biogenic incorporation of As, specifically with respect to As found in their tooth samples' sub crown dentine and enamel. Dentine has already been used as an alternative to bone samples for As measurements (Swift et al., 2015).

However, no published data on dentinal As concentrations is available for modern samples, so that currently, there is no available evidence that dentinal As concentrations do directly reflect exposure to As during life. The relationship between biogenic dentinal As concentrations and those of bones is also unclear. Therefore, further research is required to elucidate how As comes to accumulate in skeletal remains, and how this may vary between different tissues (similar to the work already performed for selected isotope ratios, e.g. O'Connell and Hedges, 2001) to allow for interpretations of As concentrations in archaeological material.

Here, we present new As concentration and bioimaging data from modern hypsodont herbivore teeth with the aim of exploring the relationship between *in vivo* exposure to As and its accumulation in dental tissues, particularly dentine. In this study, we analysed teeth from highly As-exposed, seaweed-eating North Ronaldsay sheep (*Ovis aries*) from the Scottish archipelago of Orkney, and from sheep consuming As-poor non-seaweed diets on Hoy, Orkney, and mainland Scotland. North Ronaldsay sheep naturally consume high amounts of As (about 35 mg As per day, of which over 86 % is bioavailable) as part of their regular diet of seaweed (Devalla and Feldmann, 2003; Hansen et al., 2003a). Their main foodstuff, the kelps *Laminaria digitata* and *Laminaria hyperborea*, contain around 70 µg As per g dry mass (Hansen et al., 2003b), as opposed to generally below 1 µg/g in grass (Hansen et al., 2003b; Porter and Peterson, 1975). Previous studies of seaweed-eating North Ronaldsay sheep showed elevated concentrations of As in the sheep's liver, kidney, muscle, blood and urine (Feldmann et al., 2000), and in the keratinous tissues horn (Caumette et al., 2007) and wool (Raab et al., 2002). Stable isotope ratio measurements have also shown the marked influence of the seaweed diet on skeletal tissues (Balasse et al., 2009, 2006, 2005). These seaweed-eating sheep therefore provide an ideal opportunity for documenting the results of biogenic uptake of As with respect to dentine, and other skeletal tissues.

To enable archaeologists to correctly interpret data of As concentrations in archaeological remains, this study of modern reference populations seeks to address the following questions: 1) Do As concentrations in dentine reflect the degree of (dietary) exposure to As? 2) How does As become incorporated into dentine? 3) Is there potential to differentiate between diagenetic and biogenic As in dentine by studying its spatial distribution? 4) How likely are diagenetic changes to affect dentinal As concentrations? 5) Can dentinal As concentrations be used to infer exposure to As in archaeological samples? To address these questions, we sampled teeth from sheep exposed to different amounts of dietary As, determined dentinal As concentrations by hydride generation atomic fluorescence spectrometry (HG-AFS) and created bioimages of the spatial distribution of As and other elements in teeth by laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS).

2 Materials and methods

2.1 Sample descriptions

Sheep first and second molars grow with two lobes (or lophs), each with two cusps, where each cusp contains a pulp chamber (O'Brien et al., 2014). In each of the lobes, the sides of the crown are folded into the tooth along most of the height of the tooth (Fig. 1) between the lobe's two cusps (Weinreb and Sharav, 1964). This introduces funnel-shaped cavities (infundibula), filled with tooth cementum. Sheep third molars have an additional third lobe with a single cusp, but without an infundibulum. Growth of primary dentine in sheep molars occurs in long stacked-cone-like growth layers around each pulp chamber, whereby the youngest dentine is closest to the pulp chamber (Fig. 1; Hillson, 2005; Kierdorf et al., 2013). After primary growth is completed, secondary dentine forms in each pulp chamber, reducing its size (Weinreb and Sharav, 1964). As the occlusal enamel is worn away, or when the tooth is cross-sectioned, a pattern of enamel and dentine bands in each cusp ridge, separated by the cement-filled infundibulum, becomes visible. The cone-like dentine growth layers are then worn away from the tips of the cones downwards. The crown formation of sheep first molars starts prior to birth, with crown growth completed nine months after birth, while the crown formation of second molars starts soon after birth, and the crown is completed approximately one year after birth. Third molars start crown formation one year after birth, and crown growth is completed two years after birth (Weinreb and Sharav, 1964). The incremental nature of tooth growth processes may therefore allow the acquisition of time-resolved data points.

The first set of tooth samples (second and third molars, n = 10) analysed in this study originates from a collection of mandibles gathered from North Ronaldsay sheep skeletons lying on the beach of the island of North Ronaldsay (part of Orkney archipelago) in the summer of 1988. These seaweed-eating animals of the primitive North Ronaldsay breed are thought to have died of natural causes during the preceding five years, although some may have died substantially earlier. During life, pregnant North Ronaldsay sheep are brought onto grass pastures prior to giving birth. After birth, lambs consume ewe's milk, soon supplemented by grass. After four to six months, when the lambs are weaned, ewes and lambs are brought back onto the beaches (Hillson, 2005; Upex and Dobney, 2012) where they subsist nearly exclusively on seaweed (Hansen et al., 2003a). The North Ronaldsay seaweed diet has been shown to contain three to four orders of magnitude more As than in the milk/grass diets when considering dietary uptake per kg of sheep body weight (Antunovic et al., 2005; Hansen et al., 2003a, 2003b).

The second set of teeth (third molars, n = 5) originates from a modern population of Shetland sheep grazing on grass and maritime heath in the parish of South Walls on the island of Hoy (part of Orkney archipelago). The sheep from this population were slaughtered between 1992 and 1996, and samples were taken after slaughter. As a third sample group, first and second lower left mandibular molars (n = 2) were extracted from the skull of a grass-eating sheep reared in the vicinity of the village of Bettyhill, on the northern Scottish mainland.

Sampling was performed with the aim of having as few uncontrolled differences between the sheep populations as possible. In terms of their history and physiological characteristics, North Ronaldsay and Shetland breeds are very similar (Ryder, 1983). Additionally, the sheep were all reared in broadly the same geographical area (i.e. north-east Scotland). All sampled teeth were from adults, fully formed and in wear, with exposed occlusal dentine, and infundibula still present. Second and third molars were chosen for arsenic quantification to assure seaweed-diets (in case of North Ronaldsay sheep) during tooth formation, while the spatial distribution of arsenic was studied on first and second molars which are in formation during the dietary change from grass and milk to seaweed in North Ronaldsay sheep.

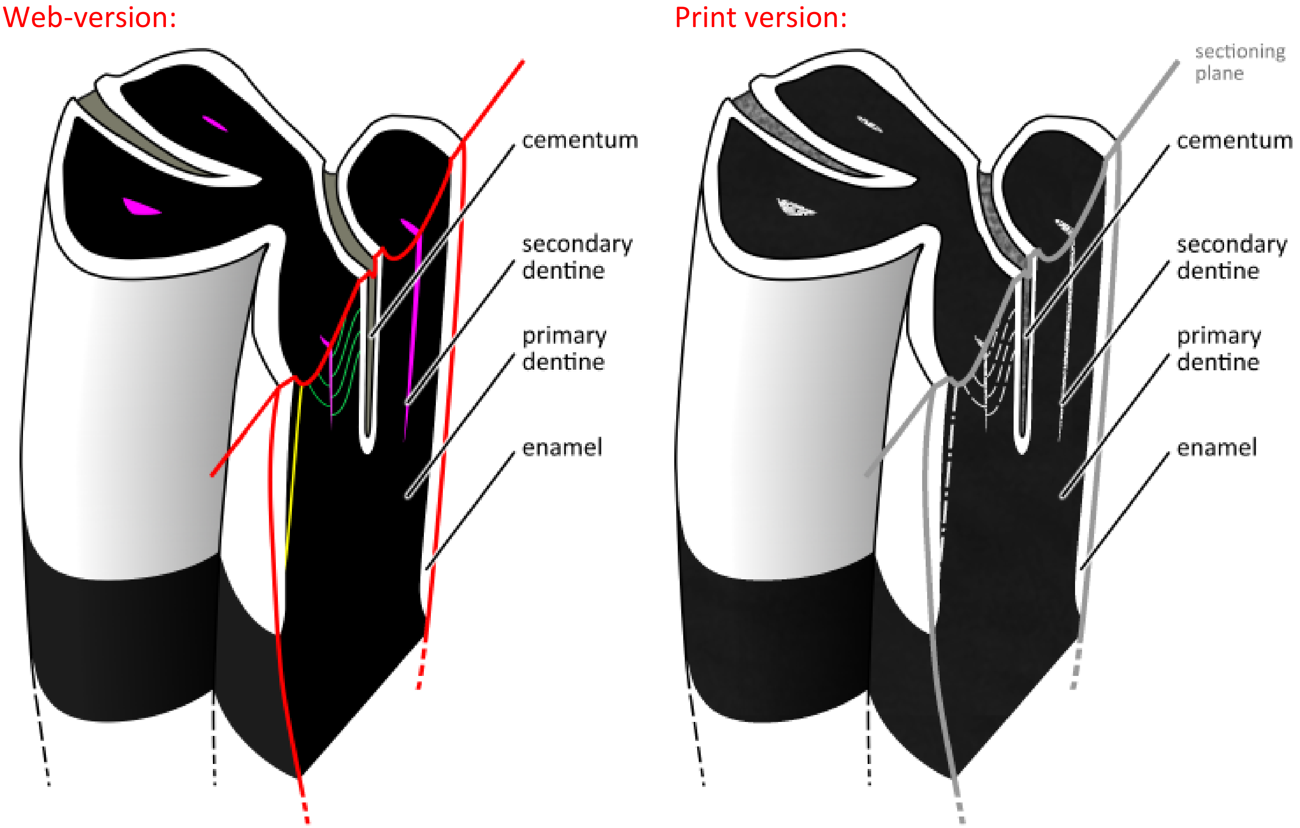

Fig. 1. *Schematic drawing of a significantly worn sheep's lower right first or second molar, occlusal to the top, radially sectioned through the middle of the mesial lobe, buccal side toward the viewer. Dashed (green in web-version) lines indicate the sinusoidal orientation of dentinal tubules in the primary dentine. The mode of growth of dentine is illustrated by the dot-dashed (yellow in web-version) line which indicates the left half of a cone-like section of dentine that was laid down simultaneously during growth of the tooth – for more detail see images in Kierdorf et al. (2013) and text. Cementum layer coating outermost tooth surface not shown here. Schematic drawing based on images and text in Every et al. (1998), Hillson (2005), Kierdorf et al. (2013), O'Brien et al. (2014), Payne (1973), Weinreb and Sharav (1964) and own observations of tooth structures.*

2.2 Quantification of arsenic by HG-AFS

After removal from mandibles, the teeth were brushed clean of surficial debris and rinsed with deionized water (19 MΩ cm, Elga, UK; used throughout experiment). Second and third molars of North Ronaldsay seaweed-eating sheep and third molars from grass-eating sheep from Hoy were prepared by removing the roots by a transverse cut using a hand-held dental drill and diamond-coated cutting discs (NTI-Kahla, Kahla, Germany), followed by ultra-sonication in deionized water. Samples of primary and secondary dentine were then obtained from both root and (internal) crown areas by drilling into the teeth from the now-exposed, cut surfaces using small tungsten carbide/diamond coated burrs (NTI-Kahla, Kahla, Germany). Sampling was performed with the consideration of preserving as much of the outer surfaces and tooth integrity as possible (enabling further studies of microwear and crown morphology), while simultaneously avoiding the inclusion of exogenous contaminants. However, because of this mode of sampling, it is possible that, in addition to dentine, small amounts of enamel and/or cementum from the infundibulum and contents of the exceedingly narrow pulp-chamber could also have been included in the sampled material. These dental cavity composite powder samples, mainly consisting of dentine, were, where necessary, further homogenised using an agate pestle and mortar. All sampling tools were cleaned with a 4 % v/v nitric acidic solution (prepared from 68 % HNO_3, analytical grade, Fisher Scientific) and de-ionised water between each sample, and dental tools were also ultra-sonicated.

Between 0.05 and 0.16 g (exact weights known) of each sample and the reference material were pre-digested in triplicate in 1 mL concentrated HNO_3 (68 %, analytical grade, Fisher Scientific). After 24 h, 1.5 mL of 30 % H_2O_2 (AnalaR NORMAPUR, BDH Prolabo) were added and the samples were then microwave digested (CEM, MARS5, Buckingham, UK) at 50 °C for 5 min, 75 °C for 5 min, and 95 °C for a final 15 min. The sample solutions were then diluted with 5 mL deionized water and analysed immediately.

Total arsenic content was measured by hydride generation atomic fluorescence spectrometry (HG-AFS, Millennium Excalibur, PS Analytical, Kent, UK) fitted with an arsenic boosted-discharge hollow-cathode lamp. The acid and reductant feeds were 3 % HCl (v/v; prepared from 32 % HCl, analytical grade, AnalaR NORMAPUR, BDH Prolabo) and 1.5 % $NaBH_4$ (m/v; prepared from $NaBH_4$ powder, Sigma Aldrich) in 0.1 mol/L NaOH (prepared from NaOH pellets, 98 %, Fisher Scientific), respectively. Argon was used as carrier gas. The arsenic standards were prepared from sodium arsenite (Merck KGaA, Germany). Further information on the HG-AFS setup is available in Rahman et al. (2000). All samples were measured in triplicate, based on triplicate sample material aliquots. Limits of detection and quantification were calculated as 3σ and 10σ of the blank, respectively. Recovery of the certified reference material human hair NCS ZC 81002b (China National Analysis Centre for iron and steel, China) with a certified value of 0.198 ± 0.023 µg/g was 81 %.

2.3 Bioimaging by LA-ICP-MS

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) uses a focused laser beam to volatilise small amounts of solid samples which are then (system-internally) transported to and analysed by ICP mass spectrometry. By combining multiple measurements on the same sample in a systematic pattern (e.g. line by line ablation of the sample surface), this mode of sampling allows for imaging of the elemental concentrations of the sample on a sub-mm scale, called bioimaging in case of biological samples (for a detailed review see Becker et al., 2014).

The samples for bioimaging were brushed clean of surficial debris and rinsed with deionised water. First and second molars from seaweed-eating North Ronaldsay sheep and the grass-eating sheep from Bettyhill were radially cross-sectioned along a buccal-to-lingual line that intersects the centres of the two distal cusps (compare sectioning plane through mesial cusps in Fig. 1) using diamond-coated cutting discs (NTI-Kahla, Kahla, Germany). The distal side of each tooth was then mounted on a glass slide using the household adhesive Blu-Tack® (Bostik Ltd., Stafford, UK).

Using a Nd:YAG laser (New Wave Research, UP-213) with a wavelength of 213 nm, the tooth samples were analysed by laser ablation coupled to an inductively coupled plasma mass spectrometer (iCAP Q ICP-MS from Thermo Scientific; argon plasma). Operating conditions may be found in Table 1. Straight, parallel lines across the teeth surfaces from the lingual to buccal side were ablated at a scan speed of 40 µm/s, with lines offset by either 0.2 or 0.4 mm. Several scan-lines were performed in the opposite direction to previous lines on several days, in order to monitor the reproducibility of the analysis, and checked for drifts in inter-element sensitivity (i.e. analyte to internal standard) by repeated ablation of the same area of the sample before and after bioimaging measurements.

In addition to ^{75}As, isotopes measured were ^{13}C (carbon) and the doubly-charged ^{44}Ca (calcium; *m/z* 22) for normalisation purposes, ^{34}S (sulphur), ^{66}Zn (zinc) and ^{208}Pb (lead) to enable comparison of their distribution to that of ^{75}As, and *m/z* 77 to enable estimation of the amount of the polyatomic interference of $^{40}Ar^{35}Cl^+$ measured on *m/z* 75. Normalisation was performed by subtracting the gas blank from all raw data, and dividing by $^{44}Ca^{2+}$ gas blank corrected intensities. Due to the current lack of matrix-matched calibration standards for LA-ICP-MS analysis of tooth tissues, no calibration was performed. All displayed bioimages are thus semi-quantitative. The collected data were used to create 2D contour graphs with the software SigmaPlot 13.0 (Systat Software Inc.), showing the ablation position on the x- and y- axes, and the ICP-MS data on the z-axis. All data points outside of the samples were manually removed. Overlays showing the underlying dental structure were drawn based on photographic images of the samples using GIMP 2.8.20 (www.gimp.org).

Table 1 *LA-ICP-MS parameters*

Operating Conditions	
Nd:YAG laser	New Wave Research, UP-213
ICP-MS	iCAP Q ICP-MS, Thermo Scientific
Wavelength	213 nm
Spot Diameter	100 µm
Scan Speed	40 µm/s
Frequency	20 Hz
Laser Energy	90 %
Resulting Average Fluency	16-18 J/cm^2
Average Energy delivered	1.3 mJ
Line Spacing	0.2 or 0.4 mm
Dwell Times:	
$^{13}C^+$	5 ms
$^{44}Ca^{2+}$ (*m/z* 22), $^{34}S^+$, $^{66}Zn^+$, $^{208}Pb^+$	10 ms
$^{75}As^+$ and *m/z* 77 for $^{40}Ar^{37}Cl^+$	500 ms

3 Results and discussion

3.1 Total arsenic concentrations of dentine samples

Using HG-AFS, total arsenic concentrations in the dental cavity composite samples, mainly consisting of dentine, of second and third molars of five North Ronaldsay seaweed-eating sheep and third molars of five grass-eating sheep from Hoy were determined. Concentrations of As in the samples of seaweed-eating North Ronaldsay sheep ranged from 0.05 µg/g to 2.94 µg/g (mean 0.88 µg/g), while similar samples from the grass-eating control population from Hoy all had As levels below the limit of detection (LOD; 0.001 µg/g). As-levels were found to be similar for second and third molars taken from the same jaw, signifying a low intra-individual variability with respect to arsenic concentrations in teeth (Table 2, and Fig. A.1 in the appendix).

Table 2 *Arsenic concentrations in dental cavity composite samples, mainly consisting of dentine, of seaweed-eating (i.e. arsenic exposed) North Ronaldsay sheep, and grass-eating sheep from Hoy. Sigma (σ) denotes the standard deviation based on triplicate measurements of three separate digestions of three sample aliquots. In case of the second molar of NR84.8b, only one measurement was made. The limit of detection was 0.001 µg/g and the limit of quantification (LOQ) was 0.003 µg/g*

Sample ID	**Sample origin**	**Main diet**	**As concentration (µg/g) ± 1σ**	
			second molar	third molar
HOY58	Hoy	grass		< 0.001
SY003	Hoy	grass		< 0.001
HOY 01	Hoy	grass		< 0.001
HOY YH53	Hoy	grass		< 0.001
HOY SY89	Hoy	grass		< 0.001
NR84.13	North Ronaldsay	seaweed	0.222 ± 0.013	0.334 ± 0.015
NRKDbox1	North Ronaldsay	seaweed	1.02 ± 0.06	1.12 ± 0.06
NR84.15a	North Ronaldsay	seaweed	0.335 ± 0.026	0.353 ± 0.022
NR84.8b	North Ronaldsay	seaweed	0.046	0.352 ± 0.025
NR84.33	North Ronaldsay	seaweed	2.94 ± 0.21	2.11 ± 0.13

3.2 Bioimaging of cross-sectioned teeth

Using LA-ICP-MS, it was possible to create several bioimages of the first molars of two seaweed-eating sheep and a second molar of a grass-eating sheep (Figs. 2 and 3). The instrument background (see Longerich et al. 1996) was around 233 ± 30 raw counts per second (values are mean ± 1σ) for As, compared to values between around 359 ± 37 counts per second in the non-occlusal dentine of seaweed-eating sheep (excluding the triangular areas with elevated As intensities).

In both seaweed-eating and grass-eating sheep's teeth, highest normalised As intensities were recorded for the infundibulum (up to 74,000 counts per second in seaweed-eating sheep) and for a triangular area of dentine at the occlusal surface. This pattern of elevated occlusal dentinal intensities was found irrespective of the degree of wear, i.e. distance from the root of the tooth was immaterial for detecting this particular pattern at the occlusal surface. Notably, elevated intensities for the infundibular cementum were also measured for lead (Pb) and zinc (Zn), but the triangular patterning visible for As in dentine was not observed in case of Pb and Zn. The essential elements carbon (C), sulphur (S), calcium (Ca) and zinc (Zn) were found to be largely homogeneous in their intensity distribution throughout each tissue type (i.e. enamel, primary dentine, secondary dentine and cementum), in contrast to As and Pb, which are discussed further below.

The on average 18-fold difference of normalised As intensities between cementum and dentine indicates a higher concentration of As in cementum than in dentine of the seaweed-eating North Ronaldsay sheep, even when taking differences in calcium (Ca) concentrations between different dental tissues (which affect normalisation) into account. The overall distribution pattern of As did not differ markedly between seaweed-eating and grass-eating sheep's teeth, but the range of measured As count rates was over one order of magnitude larger in case of the seaweed-eating sheep.

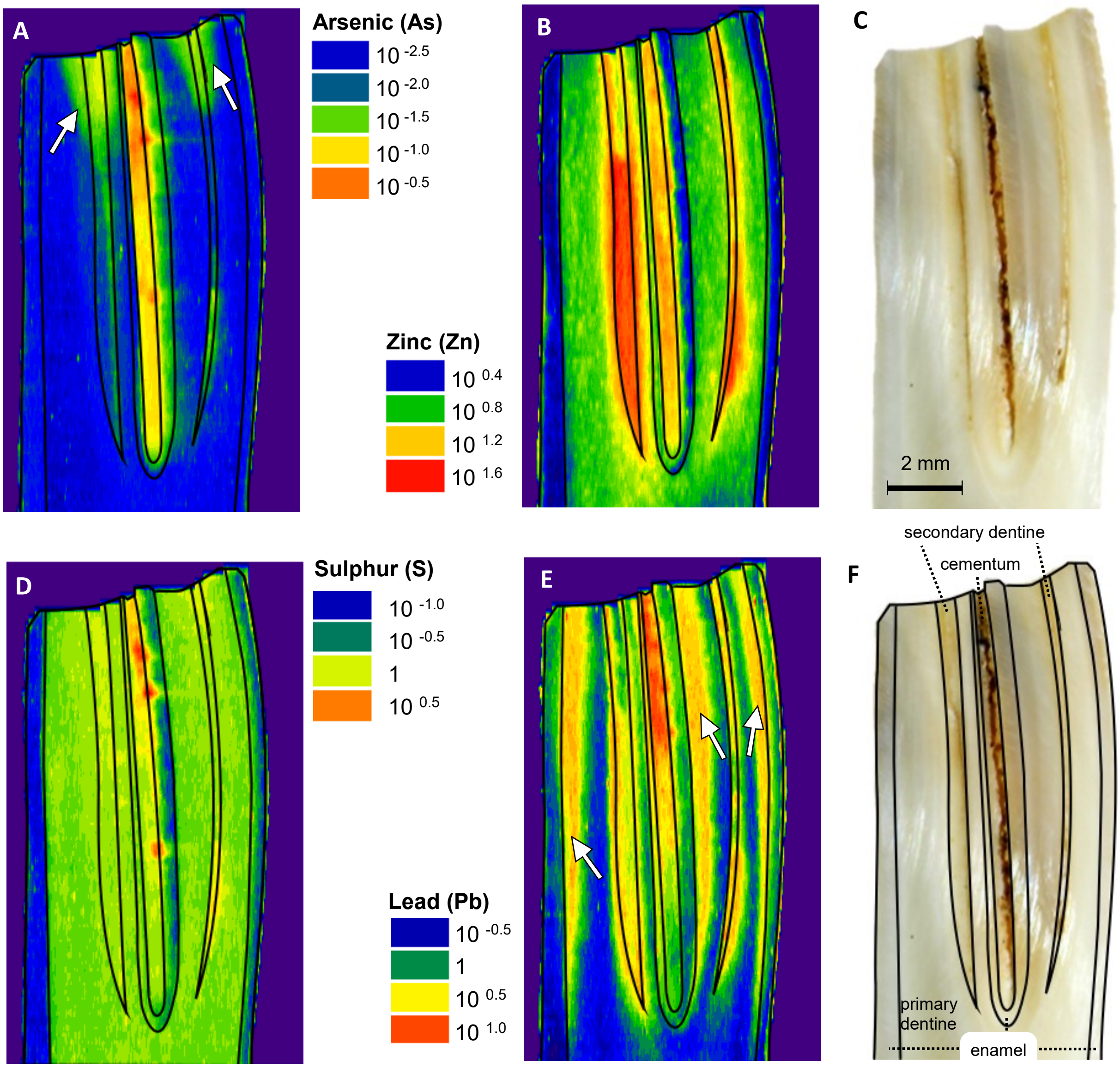

Fig. 2. *Bioimages of the distal side of a radial cross-section through the distal cusps of a first lower molar of a seaweed-eating North Ronaldsay sheep, including an overlay indicating boundaries of the different dental tissues (images A, B, D, E), and two photographs of the same tooth, with and without the overlay (C, F). The occlusal surface is facing up. Arrows in the As image (Fig. 2A) indicate triangular areas of elevated intensities at the occlusal surface; arrows in the Pb image (Fig. 2E) indicate the banded pattern of elevated intensities. For detailed description of the position of the cross-section plane, as well as dental anatomy refer to Fig. 1. Lines were ablated from left to right, causing some delayed-washout effects. Normalisation was performed to* $^{44}Ca^{2+}$*. All intensities are given on a logarithmic scale. No direct inferences of concentration differences between elements in different tissues or teeth may be drawn.*

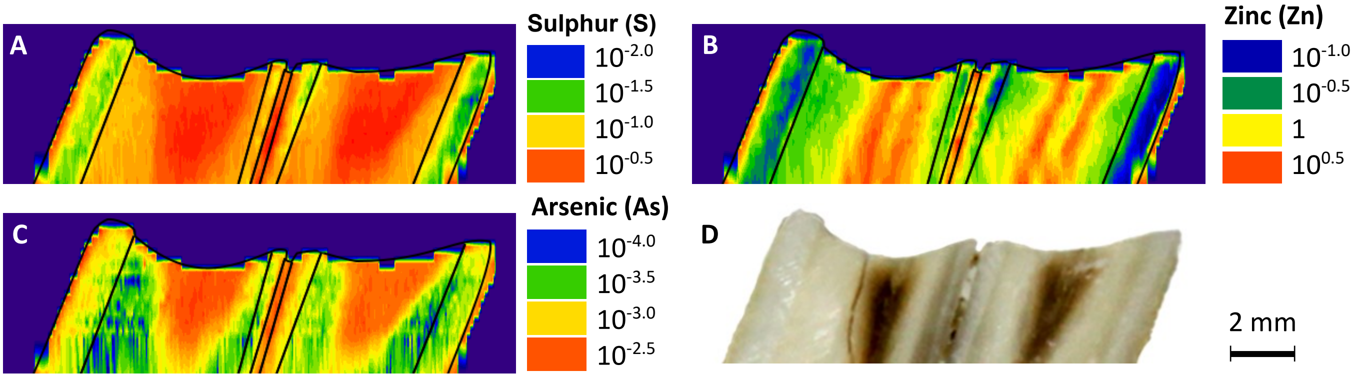

Fig. 3. *Bioimages of the distal side of a radial cross-section through the distal cusps of a first lower molar of a grass-eating sheep, including an overlay indicating boundaries of the different dental tissues (A-C; secondary dentine not outlined), and a photograph of the same tooth (D). The occlusal surface is facing up. Refer to Fig. 1 and text for detailed description of the position of the cross-section plane, as well as information on the locations of the differing tooth tissues. Lines were ablated from left to right, causing some delayed-washout effects. Normalisation was performed to* $^{44}Ca^{2+}$*. All intensities are given on a logarithmic scale. No direct inferences of concentration differences between elements in different tissues or teeth may be drawn.*

3.3 Origin of arsenic in seaweed-eating sheep's teeth

The inhalation of arsenical compounds is a known source of elevated As concentrations in bodily tissues (Rhoads and Sanders, 1985), and high As levels in drinking water have also been linked to elevated As concentrations in hair, blood and nails (Hughes et al., 2011). However, because these factors would have been fairly similar for the two groups of sheep, the presence of As in air and drinking water are unlikely to have caused such different As concentrations in the two populations in this case. Additionally, the considerably higher concentration of As in seaweed makes the contributions of air and drinking water negligible.

Since the seaweed-eating sheep's teeth may have been exposed to seawater for several years while lying on the beach, whereas the grass-eating sheep's teeth were acquired directly after slaughter, the effect of weathering by seawater also needs to be considered. According to Pike and Richards' modelling of As uptake in bone (Pike and Richards, 2002), it appears that the concentration of As commonly found in seawater (Smedley and Kinniburgh, 2002) is around one order of magnitude too low to account for As concentrations as high as 3 µg/g in bone char. Despite the limitations of the adsorption model and the differing experimental conditions, this indicates that exposure to seawater may not fully account for all As found in the infundibulum of the seaweed-eating sheep.

This leaves the sheep's diets as a possible source of origin of the elevated levels of As found in the sheep's teeth, potentially through biogenic inclusion via ingestion, or by direct contact with the tooth surface (e.g. as part of particles entering the dental tissues and cavities, by adsorption and/or by remineralisation). In case of Pb (Arora et al., 2014; Farell et al., 2013; Shepherd et al., 2012), barium (Ba; Austin et al., 2013), calcein and oxytetracycline (Kierdorf et al., 2013), it has been shown that dentine can give a spatially-resolved record of exposure to these elements/compounds during tooth formation, whereby the concentration of the compound or element in question in the dentine reflects the degree of exposure to the compound or element while this section of the dentine was formed/mineralised. Consistent with this, the change from the Pb-rich mixed milk/grass diet of lambs to the Pb-poor seaweed diet of adult North Ronaldsay sheep (Anastasio et al., 2006; Antunovic et al., 2005; Bacon et al., 1996; Hansen et al., 2003a; Najarnezhad et al., 2015; Ródenas de la Rocha et al., 2009; Schiener et al., 2015) is visible in the bioimages of our study: The arrows in Fig. 2E point out changes in Pb intensities in the primary dentine, with arrows originating in the younger primary dentine where lower intensities were observed and pointing toward the higher intensities in older primary dentine. These intensity changes correlate with the change in diet: The consumption of a Pb-rich diet at a young age is reflected by elevated Pb intensities in older primary dentine (adjacent to the enamel), and the consumption of a diet lower in Pb at an older age correlates with a change to lower intensities in the younger primary dentine surrounding the secondary dentine. (The higher Pb intensities found for secondary dentine, despite consumption of a low-Pb diet at time of secondary dentine formation, are also in accordance with the literature, which documents generally raised Pb levels in secondary dentine; Shapiro et al., 1975; Shepherd et al., 2012.) The single dietary change is reflected by multiple bands of elevated and lower Pb intensities in the primary dentine due to the cone-like growth structure of dentine on each side of the infundibulum (see Fig. 1 and *2.1 Sample descriptions*), which effectively displays the same dietary change up to four times in the same buccolingual cross-section.

However, a corresponding change in As concentration of similar or opposite patterning in primary dentine is not observable despite significant changes in the amount of dietary As. This indicates that either, unlike the case for Pb, As is not incorporated into dentine in a spatially resolved manner according to exposure to As during tooth formation, or that such an incorporation is present at very low concentrations, but not visible here due to the low overall concentration of As lowering the precision of our measurements. However, incorporation of As into the dentine at time of tooth formation seems unlikely as the cause of the elevated concentrations observed in our dentine samples.

Histologically-mediated diagenetic uptake of arsenic into teeth has been suggested to occur via the pulp chambers from the root upward in archaeological teeth (Dudgeon et al., 2016), while studies of fossils have shown dentinal tubules to contain secondary minerals as a result of precipitation, as well as submicron size clay particles (Kohn et al., 1999). However, considering that the samples in this study have not been subjected to environmental (post-mortem) diagenetic effects for a long time, but were exposed to a high-As diet throughout life, we propose the possibility of uptake of not only diagenetic material after death, but also dietary material into dentinal tubules during life.

There are several indications that saliva and noxious agents may penetrate the dentinal tubule system (Buzalaf et al., 2012; Ghazali, 2003; Götte et al., 1951; Mjör, 2009; Vernois et al., 1988). This permeability of dentine in combination with our results indicates that As may well migrate from the diet into saliva into the dentine where the enamel has been worn away, and into the cementum, bypassing the rest of the metabolism. The angle of the dentinal tubules (Fig. 1) and the decrease of dentinal permeability and circumference of the dentinal tubules from the pulp toward the enamel-dentine-juncture (Ghazali, 2003; Hillson, 2005) would then cause the triangular pattern of elevated As concentrations at the occlusal surface of the dentine (arrows in Fig. 2A). This is supported by the presence of this triangular pattern at the occlusal surface regardless of the degree of wear of the tooth, its presence at lower intensities in teeth of grass-eating (i.e. less As-exposed) sheep, and the absence of a similar pattern in non-occlusal dentine. Seawater and sea spray may also have contributed to causing this triangular pattern.

With respect to the elevated intensities measured for As in the infundibulum, the most likely explanation seems to be the direct accumulation of dietary matter. During life, cementum is deposited inside the infundibula onto the enamel, but food-debris frequently becomes trapped in infundibula (e.g. Fitzgibbon et al., 2010). The presence of this food-debris may well be the dominant cause of the considerably higher As concentrations in the cavity composite samples of the seaweed-eating sheep compared to those of grass-eating sheep, but the accumulation of arsenic in cementum by metabolic processes during cementum formation and the saliva-mediated introduction of dissolved As-containing compounds are also possibilities.

3.4 Relating exposure to As to skeletal concentrations in archaeological case studies

Regardless of whether there is a metabolic route for As to be incorporated into skeletal material, non-metabolic i*n vivo* incorporation of As can likely affect dentine and cementum in a manner similar to diagenetic alteration. Any potential incorporation of As into dentine during tooth formation according to the degree of exposure is therefore likely to be overshadowed by As taken up at the occlusal surface (whether by diagenesis or by direct contact with the diet and saliva).

Where diagenesis may be categorically excluded as a source of As, it might then be possible to use dentinal and cementum/infundibular As concentrations as indicators of dietary exposure to As. Since dentinal As concentrations are likely influenced by both the length as well as the level of direct occlusal exposure to As-rich diets, the apparent lack of temporal resolution for dentinal As concentrations implies a problem of equifinality: Both long-term exposure and recent switches to extremely As-rich or As-poor diets may lead to the same concentration and distribution of As in dentine. However, as has been shown by the study of sheep's teeth exposed to As-rich and As-poor diets presented here, dentinal As concentrations may be used as a blunt tool for investigating dietary exposure to As if dentine is exposed and diagenesis may be excluded.

The use of As concentrations in skeletal remains as direct indicators of e.g. proximity to smelting activities, deliberate poisoning, or diet (e.g. by seaweed-eating or by deliberate ingestion of arsenic oxide powder; Przygoda et al., 2001) remains problematic as conclusive evidence of the biogenic metabolic accumulation of As in human skeletal tissues directly related to the level of As exposure is currently still lacking. Multiple reference values for contemporary (at time of analysis) human bones and teeth are available (Appendix Table A.1), ranging from 0.003 to 27.3 µg/g As, with most studies reporting average values below 1 µg/g. This illustrates the broad range of biologically possible biogenic values. However, it is unclear if this range of As concentrations is caused by varying exposure to As, or other factors, such as age and the sampling of diseased tissues. Research in this field is clearly complicated by the difficulty of gaining access to samples exposed to known As levels. It is to be hoped that further experimental studies on modern materials will help to elucidate the accumulation of As in skeletal tissues, including the substantiation or rejection of claims as to the *in vivo* metabolic substitution of phosphate with arsenate in bioapatite. Only by acquiring further understanding from modern populations can As concentrations in archaeological skeletal material be interpreted adequately.

4 Conclusion

In this study, we have shown that even when exposed to high amounts of As through diet, surface contact related changes (whether these are from chewing seaweed during life or from exposure to seawater) to teeth may overprint any potential biogenic patterning with metabolic causes in the occlusal area in a very short timeframe (e.g. within a few years, and likely within the lifespan of the individual concerned). This indicates that dentine is very susceptible to diagenetic alteration by As exposure, so that where the aim is to elucidate exposure to As, dentine of potentially diagenetically altered teeth is not suitable for analysis. This supports previous reports warning that the use of As in bones as a marker for exposure to arsenic may not be viable and should be approached with caution (Pike and Richards, 2002).

If diagenesis, however, can be excluded as a possible origin of As, then it might be possible to use As in occlusal dentine as a direct indicator of dietary As. This approach is complicated by the possibility of variable dentine permeability with tooth wear, between individuals and species, and the issue of equifinality, among others. Therefore, ultimately, the results of our study are more easily interpreted as a cautionary tale for palaeodietary investigations than as a new method of identifying exposure to As.

With respect to biogenic, metabolic inclusion of As into dental tissues during the formation of the teeth, it remains unclear if the concentration of As in non-occlusal dentine reflects the individual's exposure to As. While our results confirm previous work (Arora et al., 2014; Farell et al., 2013; Shepherd et al., 2012) indicating that the spatial distribution of Pb in dentine can indeed provide a time-resolved record of exposure to Pb, the case seems to be more complicated for As: Our results indicate that either arsenic does not accumulate in dentine during the growth of the tooth in a spatially resolved manner according to the degree of exposure, or it does so only at such low concentrations that the resulting concentration differences in the exposure pattern were not resolvable by our setup. However, in this latter case, these concentration differences are likely to be negligible compared to the dentinal As concentration differences induced by diagenetic or dietary overprinting at exposed surfaces. Due to this overprinting, archaeological dentine seems to be an unsuitable sample to investigate exposure to As during life particularly when performing bulk (i.e. not spatially resolved) analyses in most cases.

For future studies aiming to measure the exposure to As by analysis of skeletal tissues, we recommend the prior study of modern populations exposed to known amounts of As in order to further investigate the assumed links between exposure and skeletal As concentrations prior to further interpretation of As concentrations in archaeological samples.

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6 Author contributions

MB reviewed the literature, performed LA-ICP-MS measurements, prepared all figures, analysed and interpreted the data and wrote and revised the manuscript; KG performed HG-AFS measurements, analysed and interpreted the resulting data and contributed to the revision of the manuscript; KB performed sampling of tooth tissues and revision of the manuscript; JF conceived the study and performed revision of the manuscript. All authors read and approved the final draft prior to submission.

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8 Appendix

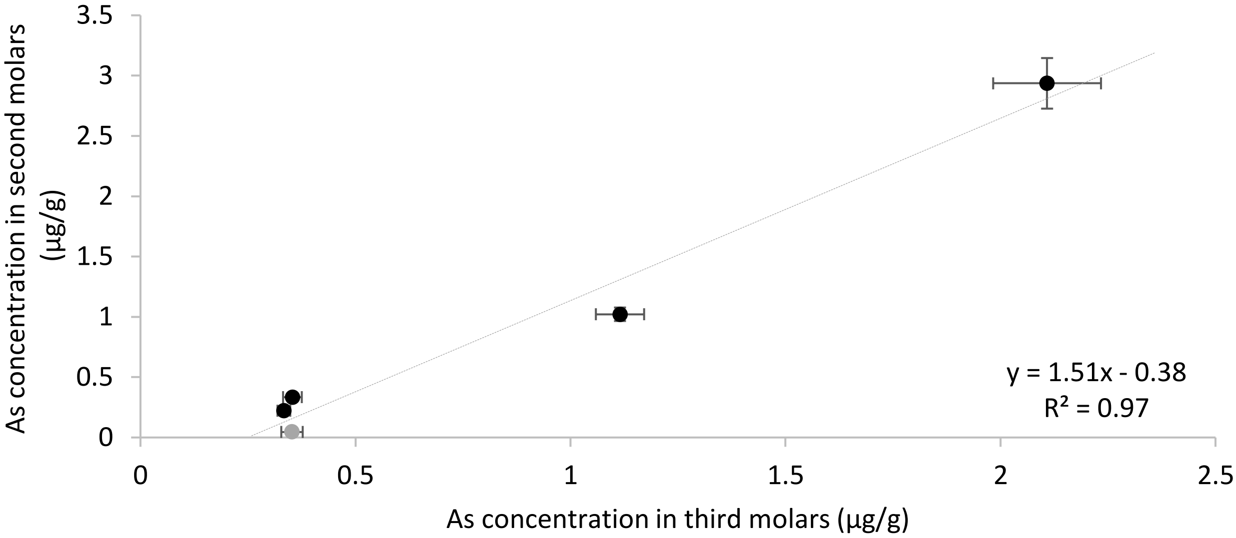

Fig. A.1 *Paired sample (single individuals) comparison for As in second molars (y-axis) and third molars (x-axis) of five seaweed-eating North Ronaldsay sheep showing linear correlation. Error bars indicate ± 1σ based on triplicate measurements. The data obtained for the second molar (y-value) of the grey marker was acquired from a single measurement only, due to small sample size. Data also shown in Table 2*

Table A.1 *Arsenic concentrations in skeletal tissues of contemporary (at time of analysis) humans reported in the literature. While efforts were undertaken towards this end, the authors make no claim as to the completeness of this table. All values shown here either refer to dry weight, or the publication did not specify whether the values referred to dry weight. Where multiple samples were taken from the same individuals, the number of individuals is given in brackets after the number of samples. As the table illustrates, current knowledge of how and why As may vary both within and between different skeletal tissues is rudimentary at best.*

mean As concentration (μg/g)	SD (1 σ)	min. value (μg/g)	max. value (μg/g)	no. samples	age of sampled population	sample type	origin of samples	health condition	exposure to As	reference
0.79		0.64	11	30		tooth roots	Saudi Arabia	chronic periodontitis	non-smoking	Alhasmi et al., 2015
0.98		0.91	1.5	30		tooth roots	Saudi Arabia	chronic periodontitis	smoking	Alhasmi et al., 2015
0.06 (ICP-MS) 0.05 (LIBS)		0.05	0.09	30		tooth roots	Saudi Arabia	no chronic periodontitis		Alhasmi et al., 2015
0.022	0.012	0.012	0.036	4		(likely whole) tooth	likely Germany	caries or periodontitis		Götte and Hattemer, 1955
0.07	0.085	0.003	0.63	75		enamel	likely UK	teeth free from enamel defects		Nixon et al., 1967
		<0.001	0.008	12	'young'	enamel	likely Denmark	teeth without fillings		Rasmussen, 1974
0.42	0.43	0.08	1.15	5		whole teeth	likely Japan	caries-free		Sairenji et al., 1962
0.14	0.07			10 (3)		enamel	Austria			Stadlbauer et al., 2007
0.11		0.03	2.33	92	average age 69.2 years	cortical bone of femur head	Poland	coxarthrosis		Brodziak-Dopierała et al., 2011
0.08		0.03	0.32	92	average age 69.2 years	trabecular bone of femur head	Poland	coxarthrosis		Brodziak-Dopierała et al., 2011
0.24				58	average age 68.2 years	femur head	Poland	coxarthrosis	non-smoking	Brodziak-Dopierała et al., 2011

0.12				34	average age 69.8 years	femur head	Poland	coxarthrosis	smoking	Brodziak-Dopierała et al., 2011
0.11						femur head	Katowice, Poland	coxarthrosis	living outside range of non-ferrous metal plant emission	Brodziak-Dopierała et al., 2011
0.22						femur head	Orzeł Biały, Poland	coxarthrosis	living within range of non-ferrous metal plant emission	Brodziak-Dopierała et al., 2011
3.0 (male) 2.6 (female)	1.6 1.3		6.9 4.8	150	12 to 87 years	bone	Korea	without special diseases	‘normal’ Koreans	Chan Yoo et al., 2002
below LOD of 0.05				78	adult	bone	Spain		living near municipal solid waste incinerator, but no occupational exposure	García et al., 2001
4.1 0.08						bone				Iyengar et al., 1978 cited in Lindh et al., 1980
3.6	0.49	<2.11	27.3	77 (70)	27.5 % aged 41-60 years; 51.3 % aged 61-80 years	bone	Taiwan	various		Kuo et al., 2000
		<0.005	0.007	5		femur	Sweden		not industrially exposed workers	Lindh et al., 1980
		0.006	0.21	7	45 to 75 years	femur	Sweden		industrially exposed workers	Lindh et al., 1980
0.32	0.12			6 (3)		femur	Austria			Stadlbauer et al., 2007
0.19	0.12	0.03	0.37	12	average age 68.0 ± 9.9	cortical part of femur head	Silesia, Poland	coxarthrosis	dust emissions of 12.5 tons/year/ km^2 As in region in 1999	Wiechula et al., 2003 & Jurkiewicz et al., 2004
0.26	0.25	0.001	0.92	13	average age 69.2 ± 9.6	cortical part of femur head	Kraków, Poland	coxarthrosis	dust emissions of 18.1 tons/year/ km^2 As in Kraków in 1999	Wiechula et al., 2003 & Jurkiewicz et al., 2004
0.43	0.38	0.08	1.42	10	average age 68.3 ± 7.3	cortical part of femur head	Łódź, Poland	coxarthrosis	dust emissions of 5.4 tons/year/ km^2 As in Łódź in 1999	Wiechula et al., 2003 & Jurkiewicz et al., 2004