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MASTER'S THESIS

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Arsenic content in the hair and nail samples of dogs living in an
old mining district and control areas in France



Zagreb, 2024



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The paper contains 65 pages, 20 figures, 8 tables, 146 literature citations.

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ABBREVIATIONS

Ar: Argon

ARS: *Agence Régionale de Santé* -Regional Health Agency (France)

As: Arsenic

As (0): Elemental arsenic

As (III): Trivalent arsenic, arsenite

As (V): Pentavalent arsenic, arsenate

As_{sug}: Arsenosugars

BASOL: *Base de données des sites et SOLs pollués* -database of polluted sites and soils (France)

BC: Before Christ

BRGM: *Bureau de Recherches Géologiques et Minières* -Geological and Mining Research Bureau (France)

BMD: Bernese Mountain Dog

BMI: Body Mass Index

BW: Body Weight

CAD: Canine Atopic Dermatitis

C: Control

CASIAS: *Carte des Anciens Sites Industriels et Activités de Services* -Map of former industrial sites and service activities (France)

CCA: Copper-Chromium-Arsenic

CKC: Cavalier King Charles

CRM: Certified Reference Material

DMA: Dimethylarsinic acid

DNA: Deoxyribonucleic acid

E: Exposed

EPA: Environmental Protection Agency, USA

EU: European Union

F: Female

g: Gram

He: Helium

IAEA: International Atomic Energy Agency

IARC: International Agency for Research on Cancer

iAs: Inorganic arsenic

ICP-MS: Inductively Coupled Plasma-Mass Spectrometry

INERIS: *Institut National de l'Environnement Industriel et des Risques* - National Institute for the Industrial Environment and Risks (France)

INRS: *Institut National de Recherche et de Sécurité* -National Institute for Research and Security (France)

INSEE: *Institut National de la Statistique et des Études Économiques* -National Institute of Statistics and Economic Studies (France)

kg: Kilogram

l: Liter

LED: Light-Emitting Diode

LOD: Limit Of Detection

LOQ Limit Of Quantification

LPS: Lipopolysaccharides

m: Meter

M: Male

Med: Median

mg: Milligram

mL: Milliliter

mm: Millimeter

m/z: Mass-charge ratio

MMA: Monomethylarsonic acid

MN: Mammary neoplasms

ng: Nanogram

NRC: National Research Council (USA)

oAs: Organic arsenic

PM₁₀: Particulate matter, particles less than 10 µm in diameter

PMD: Pyrenean Mountain Dog

ppm: Part per million

Q tr. : Quartile transformation

RMQS: *Réseau de Mesures de la Qualité des Sols* -Soil Quality Measurement Network (France)

ROS: Reactive Oxygen Species

SD: Standard deviation

SEPS: *Société d'exploitation et de Pyrométallurgie de Salsigne* -Salsigne Exploitation and Pyrometallurgy Company (France)

USA: United States of America

WHO: World Health Organization

X: crossbreed

µg: microgram

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1. INTRODUCTION

Twenty years ago, Salsigne, the last gold mine in metropolitan France, ceased operations permanently, marking the end of over a century of modern mining activity. With its closure, and despite the partial remediation efforts, concerns were raised about the mine's environmental impact and, more important, about potential risks to public health (DROUHOT et al., 2014). These issues took on greater significance following the 2018 floods, which inflicted damage upon some of the treatment facilities and storage sites in the Salsigne area, enabling the remobilization of mining waste and toxic elements from polluted sediments further downstream (KHASKA et al., 2018; GIRARDEAU, 2019; DELPLACE et al., 2022).

Despite the distinctive geological characteristics of this area, with naturally high levels of arsenic in rocks and soil, gold extraction resulted in the release of additional arsenic into the environment, along with other elements including lead, cadmium, mercury, zinc, etc. (KHASKA et al., 2018). Despite this, the consequences of mining activity on the resident population have been sparsely investigated, with no studies yet carried out on companion animals. Pet dogs have been proposed as good sentinels of long-term exposure to environmental contaminants in human population, including arsenic (VÁZQUEZ et al., 2016; CRAUN et al., 2020; HEGEDUS et al., 2023).

The aim of this study was, therefore, to analyse the arsenic levels in the hair and nails of dogs living in the former mining area of the Orbiel Valley and to compare them with those of dogs living in a control area. The hypothesis was that pet dogs' hair and nails could act as biomarkers of exposure to arsenic pollution from this ancient gold-mining area.

2. REVIEW OF THE RESULTS OF PREVIOUS RESEARCH

2.1. Arsenic overview

The term arsenic is derived from the Latin *arsenicum*, which in turn is derived from the Greek word *arsenikon* formed from *arsên* meaning potent, male (DAUZAT et al., 1971; BOWELL et al., 2014; PAUL et al., 2023). However, this is theorised to be derived from the earlier Persian *zarnik* meaning gold coloured which could refer to orpiment (BOWELL et al., 2014). It is a unique element in that it exhibits paradoxical properties, which are merely a reflection of its distinctive metabolism and characteristics.

2.1.1. Physico-chemical properties

Arsenic is a chemical element with the symbol As and the atomic number 33, which belongs to the nitrogen group of the periodic table. Arsenic is unique in that it exhibits properties characteristic of both metals and non-metals, which has led to its classification as a metalloid (JOLLIFFE, 1993; HUGHES et al., 2011; HU et al., 2020; GENCHI et al., 2022). *Arsenic metal* is a term frequently used in reference to this element; moreover, from a toxicological standpoint, it falls within the classification of metals (HU et al., 2020; GARLAND, 2021).

Native arsenic is rare and is only occasionally present in some hydrothermal base metal deposits. It is solid, heavy, fragile, and shiny steel grey in appearance (SCHARRER et al., 2020; INRS, 2023). While a minor proportion of arsenic may be present as an impurity, most of the arsenic is present in ores, predominantly associated with sulphur. These include arsenopyrite (*mispickel*, FeAsS), realgar (*arsenic blende*, *ruby sulphur*, As₄S₄) and orpiment (*yellow arsenic blende*, As₂S₃) (DICTOR et al., 2004; PROUST and PICOT, 2019). To date, more than two hundred ores containing arsenic have been identified. It should also be noted that arsenic exists in several allotropic forms and isotopes.

Nonetheless, this metalloid is ubiquitous in the environment where it is present in trace amounts as highly toxic inorganic arsenic (iAs) or less toxic organic arsenic (oAs, *organometallics*) compounds as well as in different valence or oxidation states. Those different structural forms and oxidation states are explained by its properties of forming alloys with metals and covalent bonds with hydrogen, oxygen, carbon and so forth (JOLLIFFE, 1993; HUGHES et al., 2011; DROUHOT et al., 2014; PROUST and PICOT, 2019; GENCHI et al.,

2022). Depending on its different forms, it can be transported from one medium (soil, air, water) to another more or less easily (MATSCHULLAT, 2000).

As previously stated, this metalloid exists in different valence states. Of these, the most important are positive trivalent arsenic (+III) and pentavalent arsenic (+V). Other examples include elemental arsenic (0) and negative trivalent arsenic (-III) (JOLLIFFE, 1993; CONCHA et al., 1998; PROUST and PICOT, 2019; GENCHI et al., 2022). In addition, from a toxicological and biological perspective, arsenic compounds can be classified as inorganic arsenic, organic arsenic, and arsine gas (AsH_3). Inorganic compounds, predominantly bonded to oxygen, sulphur, and chlorine, are significantly more toxic than organic compounds (although some exceptions do exist), which are themselves primarily associated with carbon and hydrogen (JOLLIFFE, 1993). It is widely acknowledged that the most prevalent arsenites are arsenic trioxide (As_2O_3 , *white arsenic*, “*the poisons of poisons*”), sodium arsenite and arsenic trichloride. In contrast, arsenates encompass arsenic pentoxide, and various other compounds (e.g. lead and calcium arsenates). Common organic arsenic compounds include arsanilic acid, methylarsonic acid, dimethylarsinic acid (cacodylic acid) and arsenobetaine (NATIONAL RESEARCH COUNCIL (US), 1999; FOWLER et al., 2015). Arsine (AsH_3 , *arsenic trihydride*, *arsane*) is regarded as the most harmful of all the arsenicals, particularly considering its primary route of absorption, which is inhalation (JOLLIFFE, 1993).

2.1.2. Arsenic of natural origin

Arsenic is naturally present in the Earth's crust where it ranks twentieth in terms of abundance (MOLÉNAT and HOLEMAN, 2000; GENCHI et al., 2022). It is therefore found throughout the ecosphere (DICTOR et al., 2004; DROUHOT et al., 2014; PROUST and PICOT, 2019). Moreover, arsenic by virtue of its nature, is known to persist in the environment (HINDMARSH et al., 1986; BELLUCK et al., 2003; BISSON et al., 2010).

Several natural processes contribute to the presence of arsenic in the environment, including the erosion of arsenic-bearing rocks, soil leaching, volcanic activity, thermal springs, and forest fires. These processes result in the release of arsenic into both the aquatic environment and the atmosphere. Subsequently, redistribution across the Earth's surface occurs via a variety of processes, including precipitation and wind (MOLÉNAT and HOLEMAN, 2000; BISSON et al., 2010; DROUHOT et al., 2014; PROUST and PICOT, 2019; SU et al.,

2023). Furthermore, scientific evidence has demonstrated the presence of arsenic in certain meteorites (PAPISH and HANFORD, 1930; HAMAGUCHI et al., 1969).

2.1.3. Arsenic of anthropogenic origin

The accumulation of arsenic in the environment is significantly influenced by human activities. Regarding the atmosphere, most of the arsenic can be attributed to fumes emitted from arsenic trioxide production industries (MOLÉNAT and HOLEMAN, 2000; INRS, 2023) and the combustion of fossil fuels (MOLÉNAT and HOLEMAN, 2000; LAPERCHE et al., 2003). The contamination of groundwater may be the result of a number of different processes. These include alterations to the bedrock caused by major public works or exploitation mining. Another potential source of contamination is the infiltration of arsenical pesticides following their widespread use (ANTONI and JAMET, 2021). It is an established fact that arsenic is not biodegradable and can therefore persist in the environment for at least several decades, even thousands of years after the cessation of such operations or the initial pollution of the environment (HINDMARSH et al., 1986; BELLUCK et al., 2003; BISSON et al., 2010; GARLAND, 2018). As proposed by MOLÉNAT and HOLEMAN (2000), the principal factors contributing to the elevation of arsenic levels in soil are the extensive use of arsenic-based products in agricultural processes, the deposition of dust produced by fossil fuel combustion, the proximity of mining operations or metallurgical foundries, and the existence of industrial waste landfill sites. While the use of arsenicals has been prohibited in most industrial sectors, there are still a few exceptions, such as the wood and semiconductors industries (BEASLY, 1999; PROUST and PICOT, 2019; GENCHI et al., 2022).

Arsenical products have been and continue to be used in some countries for pest control purposes. They are employed as insecticides, rodenticides, herbicides, and fungicides. In France, the use of arsenical compounds has been prohibited following the decree of 24 May 1973 (JOURNAL OFFICIEL DE LA RÉPUBLIQUE FRANÇAISE, 1973; SPINOSI et al., 2009; TORRES-WONG, 2018). Subsequently, until 2001, the sole authorised product was sodium arsenite in viticulture (JOURNAL OFFICIEL DE LA RÉPUBLIQUE FRANÇAISE, 2001). With regard to France, a number of chemicals derived from arsenic have been employed before the ban in agricultural practices, particularly for the treatment of potatoes, as well as in arboriculture and viticulture. However, other crops including beets and olive trees could have been treated with such products (SPINOSI et al., 2009; BISSON et al., 2010; TORRES-WONG, 2018). Derivatives of arsenic have also been utilised in the context of woodworking. France has

prohibited the use of CCA (Copper-Chromium-Arsenic) as a wood preservative since 2004, except for few specified cases (SUBRA et al., 1999; LARIGNON and FONTAINE, 2018). In addition to their use in agriculture and woodworking, arsenic derivatives were used in leather production as tanning agents and in taxidermy for their insecticidal properties, a practice that persisted in some instances until the end of the 20th century (MITHANDER et al., 2017; GRAHAM 2018; TORRES-WONG, 2018).

Arsenic compounds were well-established in glass production, used for both bleaching and refining as well as in the production of mirrors (ALLEN and ZIES, 1918; IARC, 1993). In metallurgy, they were used primarily as an additive or coating agent (CARAPPELLA, 2002). Despite the low levels of exposure to arsenic through these sources for both human and animal populations, the presence of arsenic has been identified in the dust and immediate environment surrounding the aforementioned factories (CARAPPELLA, 2002; PROUST and PICOT, 2019). It is acknowledged that exposure can occur in other contexts, such as the construction industry. This is particularly the case for renovation and demolition work, due to the presence of paints containing arsenic-based pigments, including *Paris green* (copper acetoarsenite), as well as the presence of arsenic coating on metal (TORRES-WONG, 2018; GENCHI et al., 2022). Furthermore, *Paris green* was employed in fireworks as it created a deep blue coloration that could not be replicated. It was also used as a pigment alongside other arsenicals for various mediums, including book covers and wallpapers (CHEVALIER, 1859; SAXE et al., 1964; BEASLY, 1999; GAZIN, 2023).

However, in the present era, the most significant sources of exposure are ancient and contemporary mining operations and the associated pollution. The extracted minerals are employed in a diverse range of applications, including the production of electronic devices. In addition, arsenic can also be obtained as a by-product of the processing of a range of ores, including but not limited to those containing gold, cobalt, lead and copper (CARAPPELLA, 2002). When discussing coal mining for use in the power industry, it is important to note that arsenic is present in coal in varying concentrations. As a result, the use of this fossil energy source is a significant contributor to atmospheric arsenic contamination (MOLÉNAT et HOLEMAN, 2000; PROUST and PICOT, 2019; GENCHI et al., 2022; SU et al., 2023). Moreover, the incineration of waste, including electronic devices, may also constitute a potential source of exposure. This is due to the use of arsenic in the semiconductor industry and its presence in a range of electronic components, including LED (Light-Emitting Diode) lights,

laser printer drums, photovoltaic cells, and so-called *smart electronics* (PROUST and PICOT, 2019; GENCHI et al., 2022).

2.1.4. Sources of exposure

Arsenic can be derived from a multitude of natural and anthropogenic sources and will ultimately be incorporated into the environment through biogeochemical cycles (SU et al., 2023).

The concentration of arsenic in the atmosphere is typically low, with levels generally below 10 ng m^{-3} (nanograms per cubic meter), and similarly low concentrations are found in surface water and groundwater, with levels ranging between $1\text{-}10 \text{ }\mu\text{g l}^{-1}$ (micrograms per liter). Arsenic is of particular significance in soil, where it can reach levels of 100 mg kg^{-1} (milligrams per kilogram) and higher, dependent on the geological zones in question (MOLÉNAT and HOLEMAN, 2000; DROUHOT et al., 2014; ANTONI and JAMET, 2021). For example, regions in France exhibiting elevated levels of arsenic (Figure 1) are primarily situated in proximity to bedrock formations but also in ancient volcanic zones (RAVAULT et al., 2002; ANTONI and JAMET, 2021). A positive correlation has been identified between the concentration of arsenic in soil samples and that in water, including groundwater (MIQUEL, 2003; PICOT et al., 2013).

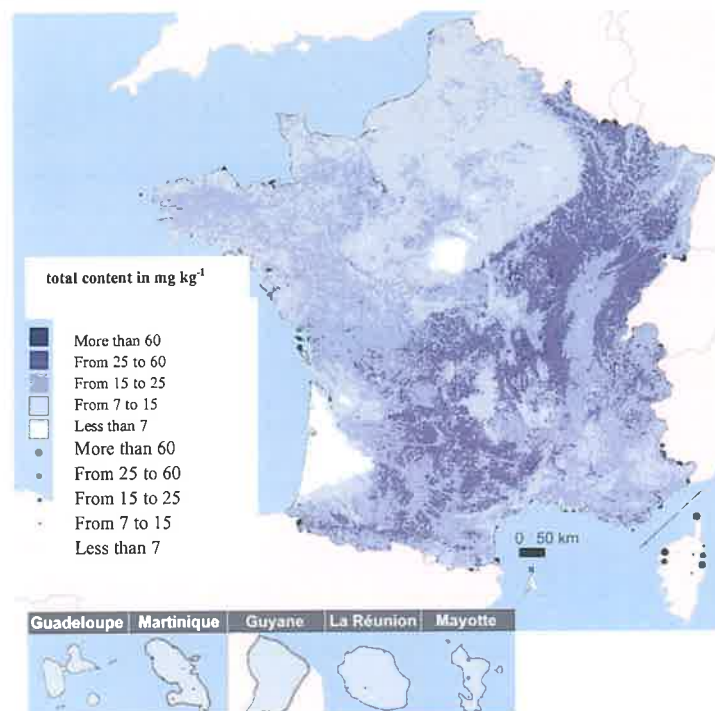


Figure 1: Arsenic levels in superficial soils (GIS SOL and RMQS (2021), adapted from LOISEAU et al. 2021; SDES, 2021)

A study conducted in 1997 to assess the arsenic contamination of water intended for human consumption in various departments of mainland France revealed the presence of arsenic concentrations exceeding the specified threshold of $10 \mu\text{g l}^{-1}$ in several locations (RAVAULT et al., 2002). A concentration of arsenic in drinking water exceeding 0.25 ppm ($250 \mu\text{g l}^{-1}$) is believed to have the potential to induce toxic effects, particularly in larger animals (GARLAND, 2021). In France, the legal limit for arsenic concentration in drinkable water has been reduced from $50 \mu\text{g l}^{-1}$ to $10 \mu\text{g l}^{-1}$ since 2001. This follows a European directive and a recommendation from the World Health Organization (WHO) (MANLIUS et al., 2009; BISSON et al., 2010).

It is of significant importance to acknowledge that inorganic arsenic is the predominant form of arsenic found in the water of specific geographical regions (Taiwan, Bangladesh, certain regions of India, and Argentina) as well as in certain food products, including rice. This contrasts with organic arsenic, which is commonly present in fish and seafood (MEAD, 2005; PROUST and PICOT, 2019; ROSENDAHL et al., 2020).

Food is an equally important source of arsenic. Though arsenic is generally regarded as being absorbed by all plants, scientific evidence has demonstrated that there is a notably elevated concentration of this element in leafy vegetables, rice, apple, and grape juice, as well as seafood (GENCHI et al., 2022). Furthermore, it is evident that the preparation of foods in water containing arsenic will result in an increase in the arsenic content of the foods in question. Similarly, irrigation waters with elevated arsenic concentrations can significantly increase the arsenic content of rice and vegetables (MEAD, 2005). In a number of countries in Europe, Asia and also in the USA, the average dietary exposure to inorganic arsenic in humans ranges from 0.1 to $3 \mu\text{g As kg}^{-1}$ body weight (BW) per day (NURCHI et al., 2020).

The bioaccumulation of arsenic in living organisms is dependent on trophic levels. Marine organisms, such as fish, molluscs, and crustaceans, are particularly susceptible to this process. Consequently, seafood frequently exhibits elevated arsenic concentrations, with levels reaching approximately $10\text{--}100 \text{ mg kg}^{-1}$ dry weight. However, most of this arsenic is organic, in the form of arsenobetaine. Arsenobetaine is considered non-toxic and does not undergo metabolic transformation within the body (POPOWICH et al., 2016; TAYLOR et al., 2017; PROUST and PICOT, 2019; GENCHI et al., 2022). In light of recent research, the potential toxicity of a class of lipid-soluble arsenic species, known as arsenolipids, has been the subject of investigation. The predominant form of arsenic present in seaweeds is that of arsenosugars

(As_{Sug}), which are ribose derivatives and readily absorbed by the gastrointestinal tract. Arsenosugars have been demonstrated to lack acute toxicity; however, there is a potential for chronic toxicity (MAC MONAGAIL and MORRISON, 2019; GENCHI et al., 2022).

The exposure of humans and animals can be attributable to several factors, including the ingestion of contaminated food and water, the use of therapeutic agents as well as occupational exposure in humans (IARC, 2004; HU et al., 2020; SU et al., 2023). For the general population and companion animals, food represents the primary source of this metalloid (NATIONAL RESEARCH COUNCIL (US), 1999; IARC, 2004). Nevertheless, water may be a significant source of arsenic exposure, especially in some geographical regions where arsenic concentrations in drinking water are markedly elevated (PROUST and PICOT, 2019; SU et al., 2023). Conversely, accidental exposure in animals, resulting from the ingestion of commercial and home-made poisoned baits and the utilisation of pesticides, although less prevalent in the current era, persists (BEASLY, 1999). Furthermore, past industrial activities have also resulted in the release and extensive dispersion of both naturally occurring and anthropogenic-derived compounds in the environment, with the potential to adversely affect wildlife, humans, and companion animals (DROUHOT et al., 2014). Susceptible individuals may be exposed by inhaling contaminated air and dust, as well as by consuming food, water, and soil from former mining areas (DROUHOT et al., 2014).

2.1.5. Arsenic in medicine

The golden era of arsenicals as therapeutic agents begun at the conclusion of the 18th century when the therapeutic effects of a solution initially referred to as *liquor mineralis* (*Fowler's solution*, KAsO₂ 1% potassium arsenite solution) were discovered. During the 19th and 20th centuries, novel arsenic-based drugs and solutions were developed (JOLLIFFE, 1993), particularly for the treatment of various cancers and trypanosomiasis (*sleeping sickness*, *Nagana*) (JOLLIFFE, 1993; HUGHES et al., 2011; FAIRLAMB and HORN, 2018; DE KONING, 2020; GENCHI et al., 2022). Nevertheless, most of these pharmaceutical agents have been withdrawn from use due to their adverse effects, variable efficacy, and the availability of safer alternatives (JOLLIFFE, 1993; BRADOL and VIDAL, 2011; NADAR et al., 2019). However, research is still in progress, as derivatives of arsenic have shown potential as antiviral agents and therapeutics for some types of cancer as well as against multi drug resistant organisms (NADAR et al., 2019; PAUL et al., 2023).

In veterinary medicine, organic arsenicals were commonly used as feed additives for livestock, particularly chickens and swine, even after their use was no longer endorsed in human medicine (JOLLIFFE, 1993; SHARMA and ANAND, 1997; PAUL et al., 2023) due to their observed anti-parasitic, growth-promoting, appetite-enhancing and digestive-stimulating properties (HOEKENGA, 1951; MCDOUGALD, 1979; JOLLIFFE, 1993; ZHAO et al., 2020; PAUL et al., 2023).

2.1.6. Toxicokinetics

2.1.6.1. Absorption

The primary route of entry is by ingestion. The absorption of arsenicals is subject to several factors, including the form of the metalloid, solubility, particle size, purity, species affected, and the physical condition of the exposed animals. Consequently, the estimation of lethal doses presents a significant challenge (BEASLY, 1999; GARLAND, 2021).

The literature indicates that in humans and experimental animals, approximately 80-90% of a dose of arsenite or arsenate is absorbed at the gastrointestinal level (BEASLY, 1999; NATIONAL RESEARCH COUNCIL (US), 1999; BISSON et al., 2010). The majority of poorly soluble arsenic compounds, such as arsenic selenide and arsenic trisulfide, are excreted mostly unchanged in the faeces (BEASLY, 1999; NATIONAL RESEARCH COUNCIL (US), 1999).

Another potential route of entry is the respiratory route, which is more prevalent in humans compared to companion animals. Inhalation of arsenic can occur in the gaseous state, although it is mainly absorbed in the form of particulate matter. It is estimated that the rate of absorption by the respiratory route in humans is equal to 30–34% (ATSDR, 2007; BISSON et al., 2010).

Of all potential routes of absorption, the transcutaneous route has been the least studied in both human and veterinary medicine. The current literature on this topic is limited, with most studies focusing on human subjects where it is regarded as a relatively minor route of absorption (ATSDR, 2007).

2.1.6.2. Distribution

Once absorbed into the bloodstream, arsenic strongly binds to plasma proteins and haemoglobin. A substantial part of an ingested low dose of arsenic will be quickly removed from blood and excreted in urine over the following hours or days depending on the species. The situation is markedly different when exposure is chronic (PRATT et al., 2016; GARLAND, 2018). While some of the accumulated arsenic may be cleared from the body over time, a part will be retained in keratin-rich tissues, where it could be detected approximately two weeks after exposure (PRATT and al., 2016). A review of the existing research suggests that arsenic distributes to all organs at varying speed during the initial exposure. The liver and kidneys appear to be the organs most affected, with the potential to accumulate significant quantities of arsenic that will be removed over time. In contrast, it is less pronounced in muscles and lungs (GARLAND, 2018).

However, arsenic compounds can also accumulate in high concentrations in the bone as well as in other keratinised tissues, including skin, hair, hoof, and nail tissues where they will be stored without clearance for a considerable length of time (ATSDR, 2007; GARLAND, 2018). This can be explained by a high affinity of As (III) for sulfhydryl groups (GENCHI et al., 2022). The highest retention in humans occurs in hair and nails, with concentrations ranging from 0.02 to 1 mg kg⁻¹ of dry weight (NATIONAL RESEARCH COUNCIL (US), 1999). Studies in humans indicate that the inorganic form accumulates with age. Moreover, it is well documented that inorganic arsenic readily passes the placental barrier in humans (TORRES-WONG, 2018) as well as in other mammals, particularly monkeys, hamsters, and gerbils (GARLAND, 2018)

2.1.6.3. Metabolism

As GARLAND (2018) observes, the biotransformation of arsenicals remains a poorly understood area of research. The liver is the primary site of arsenic metabolism in mammals. However, some compounds can undergo metabolism in other tissues, including the kidneys and lungs. For example, the kidneys may facilitate the reduction of a small quantity of pentavalent arsenic to a more toxic trivalent form (GARLAND, 2018; GENCHI et al., 2022). In addition to oxidation-reduction reactions, methylation has a role in arsenic metabolism, leading to the production of methylated metabolites such as MMA (monomethylarsonic acid) and DMA (dimethylarsinic acid). Methylation principally occurs through a process mediated by soil

microorganisms, although inorganic arsenicals are also methylated *in vivo* (GARLAND, 2018; TORRES-WONG, 2018). It is possible that this methylation of iAs could be a form of activation process that may lead to the formation of more reactive species with specific toxic properties (DROBNA et al., 2009; GARLAND, 2018; TORRES-WONG, 2018). Inter-individual differences in methylation capacities are of particular importance in this context. Such differences may be partially responsible for the observed variation in susceptibility to arsenic poisoning. It should further be emphasised that environmental, behavioural and dietary factors in humans and potentially in other mammals can influence toxicity (JANSEN et al., 2016).

2.1.6.4. Elimination

As a rule, inorganic arsenic and its metabolites are eliminated through the urine and faeces (via bile). Other minor routes of excretion exist in humans and include sweat and desquamation (shedding of skin cells) (ATSDR, 2009). In humans, arsenic metabolites are typically quantified as follows: 10-30% inorganic arsenic, 10-20% MMA, 60-80% DMA (NAVAS-ACIEN et al., 2009; JANSEN et al., 2016). These numbers are relatively consistent across a range of species that have been studied (DROBNA et al., 2009). In most human cases, between 40% and 70% (45-75% according to PRATT et al. 2016) of the absorbed quantity of pentavalent arsenicals are excreted through the urine within 48 hours while smaller amounts may be excreted through sweat. In animals, the excretion process for trivalent forms of arsenic is slower and occurs through the bile into the faeces (BEASLY, 1999; GARLAND, 2018). In contrast, arsenosugars and arsenobetaine, which are respectively found in algae and seafood are both excreted in urine (GENCHI et al., 2022). Lastly, there is evidence from multiple studies that arsenic can be excreted in human breast milk (CONCHA et al., 1998; SAMIEE et al., 2019).

2.1.6.5. Mode of action

Inorganic and organic arsenic display a distinctive affinity for sulfhydryl groups (-SH). In particular, arsenites have been observed to readily bind with these groups and thus have the potential to inhibit sulfhydryl-containing enzymes. Because of the inhibition of critical enzymes, there could be a disruption to both pyruvate oxidation pathway and Krebs cycle, impaired gluconeogenesis, and a reduction in oxidative phosphorylation (BEASLY, 1999; BERGQUIST et al., 2009; ATDSR, 2007; GENCHI et al., 2022). Arsenic exerts effects at the mitochondrial level with increased synthesis of reactive oxygen species (ROS), lipid peroxidation, protein and DNA damage (BERGQUIST et al., 2009; GENCHI et al., 2022; SU

et al., 2023). ROS production could elicit a range of modifications in cellular behaviour, including alteration of signalling pathways and epigenetic modifications, as well as direct oxidative damage to molecules (BERGQUIST et al., 2009; HU et al., 2020). In addition, the replacement of phosphate ions with iAs (V) has the potential to disrupt several other chemical reactions (GARLAND, 2018; GENCHI et al., 2022). Other mechanisms of toxicity may include autophagic defects and inflammation (HU et al., 2020).

Some arsenical compounds have been demonstrated to induce vascular instability (BEASLY, 1999; GARLAND, 2018). Arsenic is also a potent endocrine disruptor and is capable of altering hormone-mediated cell signalling when present in extremely low concentrations (MEAD, 2005). Organic arsenic compounds display a high affinity for selenol groups (-SeH) present in thioredoxin reductase and glutathione peroxidase enzymes, which may account in part for the oxidative stress commonly associated with arsenic poisoning (NURCHI et al., 2020; GENCHI et al., 2022).

Lastly, a variety of epigenetic alterations in mammalian cells, both in vivo and in vitro, can be induced by arsenic. These changes may potentially contribute to the onset of diverse forms of cancer (GENCHI et al., 2022). Arsenite, in contrast to arsenic, has been shown to possess weak mutagenic properties, and is able to potentiate the mutagenicity of other carcinogens (SU et al., 2023). Despite the classification of arsenicals as carcinogens in humans, this has not been substantiated in animal models. Attempts have been made to document arsenic-related cancer in animals, yet these experiments have yielded inconclusive results (GARLAND, 2018).

2.1.7. Toxicity

Since 1980, the International Agency for Research on Cancer (IARC) has classified arsenic and arsenicals as carcinogenic to humans. Arsenic was among the first compounds to be identified as carcinogenic by the IARC (Group 1), the Environmental Protection Agency (EPA) (Class A) and the European Union (four arsenicals are classified in the first category). Arsenic has been shown to be a clastogenic agent both in vitro and in vivo (BISSON et al., 2010).

The different forms of arsenic have varying effects on the body in both humans and animals, leading to different toxic disease syndromes and, consequently, different clinical pictures. Inorganic and trivalent arsenicals primarily affect the gastrointestinal tract and

capillaries, whereas pentavalent organic arsenicals are known to result in the development of a neurological syndrome (BISSON et al., 2010; GARLAND, 2018). GARLAND (2021) posits that the solubility of the compound, the species of animal involved, and the duration of exposure are significant factors that should be thought about when considering the toxicity of various arsenicals. This toxicity can be ascribed to their reactivity with sulfur-containing compounds, which gives rise to the generation of highly reactive oxygen species (JOLLIFFE, 1993; HU et al., 2020; GARLAND, 2021). Therefore, a ranking of the relative toxicity can be established as follows: (I As⁺³ > I As⁺⁵ > O As⁺³ > O As⁺⁵ > As⁰) inorganic As⁺³ (arsenite) has the greatest toxicity, followed by inorganic As⁺⁵ (arsenate), then trivalent organics and pentavalent organics. It is accepted that elemental arsenic is nontoxic even when ingested in considerable quantities (JOLLIFFE, 1993; GARLAND, 2018).

With regard to species, the greatest susceptibility to inorganic arsenicals is observed in humans, followed by canines and rodents (GARLAND, 2018). However, SELBY et al. (1977) indicate that dogs and cattle are the most susceptible to arsenic intoxication when compared to other species, while few cases of poisoning have been proven in cats, horses, and pigs. BEASLY (1999) on the other hand, specifies that poisoning is most prevalent in bovine and feline animals, while poisoning of dogs is occasional. Acute arsenic poisoning is the most frequently seen and documented form in dogs while chronic intoxications appear to be more frequent in cattle (SELBY et al., 1977). Animals of advanced age, younger and debilitated animals are particularly susceptible to the effects of arsenic poisoning. Peracute and acute toxicosis are often characterised with both high morbidity and mortality over two to three days (SELBY et al., 1977; BEASLY, 1999; GARLAND, 2021).

Inorganic arsenical toxicosis is typically an acute condition. Although less common, peracute poisoning may also occur. The gastrointestinal tract and cardiovascular system are the primary organs affected by this form of toxicity (SELBY et al., 1977; GARLAND, 2021). In the case of subacute arsenic poisoning, animals may survive for several days before succumbing to the disease, during which time they may display a range of clinical signs related to the gastrointestinal and urinary tracts, as well as the vascular and neurological systems (SELBY et al., 1977). Chronic exposure (*arsenicosis*) to arsenic has been rarely observed in animals but has been well documented in man (BEASLY, 1999).

Various animal studies have corroborated the adverse effects of arsenic exposure observed in humans, such as cardiotoxicity, neurotoxicity, and immunotoxicity (SU et al.,

2023). Furthermore, evidence indicates that exposure to arsenic, particularly iAs can induce ulcerative dermatitis, liver damage, chronic renal disease, as well as act as a causative agent for myocarditis in dogs (KIM et al., 2018). GARLAND (2018) suggests that animals suffering from *arsenicosis* will present with dyspnoea, intense thirst, and a general state of weakness. Moreover, they will exhibit abnormalities in their hair coat and mucous membranes, which may appear dry and brick-red in colour.

2.1.8. Diagnostics in veterinary medicine

In the context of acute arsenic toxicity, it is important to diagnose it as fast and accurately as possible, given that it enables the delivery of appropriate care and increases the likelihood of a positive survival outcome. The final confirmation of inorganic arsenic toxicosis is provided by the determination of arsenic concentration in tissues, specifically the liver, kidneys, or stomach contents (GARLAND, 2018). BEASLY (1999), indicates that both gastrointestinal contents and urine may be useful in identifying the route and degree of exposure. However, it should be noted that the concentration of arsenic in urine can remain high for several days after ingestion due its metabolism (BEASLY, 1999; GARLAND, 2021). Characteristic gross lesions as well as pathohistological changes may also be helpful (SELBY et al., 1977).

In contrast, chronic exposure is considerably more challenging to diagnose, given that arsenic-related illnesses often manifest over an extended latency period, potentially years to decades after initial exposure for humans and many patients may remain asymptomatic for years. However, the use of hair and nails as biomarkers of arsenic exposure in both animals and humans has been proposed as a non-invasive method of evaluating chronic arsenic toxicity. Hair analysis provides an assessment of the deposition of elements over a minimum period of two to three months (as hair grows approximately 1 cm per month) within its cells and interstitial spaces. Hair can be transported with minimal effort and stored without a loss of pollutants for an extended period of time (ATDSR, 2007; VÁZQUEZ et al., 2013; KATZ, 2019). Consequently, the use of pet dogs in epidemiological studies is of particular benefit, particularly when investigating contamination by toxic metal(loid)s. Pet dogs may be regarded as valuable sentinels, given their shared environment with humans. Additionally, pet dogs respond in a manner similar to that of humans to many toxic insults, and have a shorter life span, developing the consequences of some of these exposures more quickly than humans (BACKER et al., 2001). Though data is lacking, arsenic compound ratio analysis in human hair showed that up to 98% is present in form of inorganic arsenic (with low and highly varying amounts of DMA

and MMA (KATZ, 2019). In addition, arsenic levels in hair of unexposed human adults are generally below 1 mg kg⁻¹ dry weight (WHO 1983; HINDMARSH, 2002; ATSDR 2007; WU and CHEN, 2010) and there is little doubt that levels above 1 or 2 ppm are indicative of chronic exposure (KATZ, 2019). Arsenic in human toenails were shown to correlate with hair and fingernail concentrations, as well as with urine levels mainly among highly exposed populations having mean/median toenail As equal or higher than 1 µg g⁻¹ (SIGNES-PASTOR et al., 2021). However, there is no well-defined international standard for the "normal" level of arsenic species in human hair, much less in pet dogs (KATZ, 2019).

2.2. The Orbiel Valley and the former mining-district of Salsigne

2.2.1. Geological and hydrological background

The Orbiel Valley is located on the southern slopes of the Montagne Noire Mountain range, where the Orbiel River originates. The Valley experiences a continental climate, influenced by both Mediterranean and oceanic systems, leading to high annual rainfall (900 mm), mild average temperatures (13°C), frequent winds (over 300 days per year), and flash floods (DELPLACE, 2022). The valley's lithology is highly variable, with ore deposits occurring as veins or layers, ranging from surface-level to deep underground (VERMEERSCH, 2012). The Salsigne district is notable for its sulphide mineral accumulations, primarily containing iron, copper, gold, as well as arsenic and bismuth (PÉREZ and VALIENTE, 2005; DELPLACE, 2022). Consequently, the area naturally has elevated arsenic levels in the soil, surface water, and groundwater. Arsenic enters the water through natural weathering and leaching of arsenic-bearing minerals (ASSELIN and SHAW, 2016). The Grésillou and Villanière streams join the Orbiel River (Figure 2), while other important streams like the Ru Sec, Malabau, and Gourg Peyris also contribute to the flow. These watercourses carry both natural and anthropogenic arsenic to the Aude River, which, along with other rivers impacted by former mining activities (such as the Orb, Hérault, and Gardon rivers), eventually discharges into the Mediterranean Sea (ELBAZ-POULICHET, 2017).

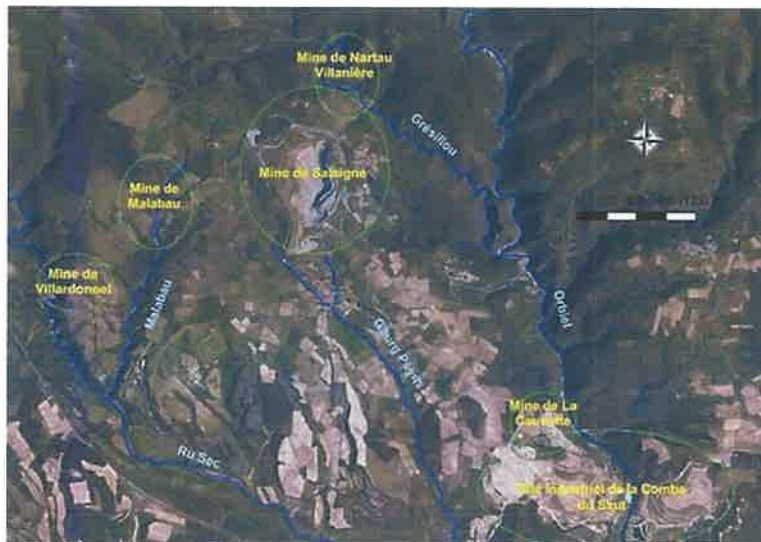


Figure 2: Hydrological context and main mining operation locations in the district of Salsigne (GIRARDEAU, 2019; reproduced by kind permission of the BRGM)

2.2.2. Former mining activities in the district of Salsigne

Although arsenic occurs naturally in the region, human-induced activities have markedly influenced its concentration levels. Mining operations can release arsenic to the atmosphere, to soil, or to water through various mining-related activities, including drilling, blasting, transportation, comminution, roasting, smelting, leaching (and associated solution handling), and storage of waste rock and tailings (ASSELIN and SHAW, 2016). Mining operations in the Orbiel Valley and Salsigne have their origins as early as the 2nd century BC (VERMEERSCH, 2012) and included iron, copper, lead, and silver mining (SAUZAY, 2004). The discovery of the gold ores in 1892 marked the beginning of a notable period of expansion within the region's mining industry. Arsenic commonly occurs in gold-bearing minerals, so processing of gold was inseparably connected to consequence of disposal of large quantities of As-containing waste (ASSELIN and SHAW, 2016). At some point during the 20th century, the main mine in Salsigne became the primary gold producer in Europe and the largest arsenic producer globally (DROUHOT et al., 2014). Another type of pollution was the result of the processing of toxic industrial waste of various origins by the SEPS (Société d'Exploitation et de Pyrométallurgie de Salsigne) during the 1990s. Gold was extracted over the decades using both pyrometallurgical and hydrometallurgical methods, the latter using cyanide (PRÉFET DE L'AUDE, 2021). As a consequence of the mining and processing of ores, the surrounding industrial plant and exploitations have been significantly contaminated by arsenic, primarily in its pentavalent form (V). This has occurred as a result of the emission of slag, fumes, and dust

(DROUHOT et al., 2014). The Salsigne mining district included various concessions and multiple extraction sites (Figure 3) until 2004 when mines were closed. The French Senate published a report in 2003 identifying the Salsigne mine, operational at the time, as the most prominent example of historical pollution in France (MIQUEL, 2003; COUR DES COMPTES, 2006; PROUST and PICOT, 2019). Various remediation actions conducted at La Combe-du-Saut from 1996 to 2006 resulted in mosaic of contaminated and rehabilitated zones (DROUHOT et al., 2014). Several millions of tonnes of waste were stored with variable safety regarding the leaching of toxic metal(loid)s to the environment at three main waste storage sites (Montredon, Artus, La Combe-du-Saut) and several others (Nartau, Malabau, La Caunette, Salsigne) (CALAS et al., 2024). Around 5000 inhabitants live in the Salsigne district today mostly in small towns (population up to 2575 in Conques-sur-Orbiel; INSEE, 2021) with the biggest city Carcassonne (population of 46218; INSEE, 2021) located on the Orbiel to Oude confluence in the south.

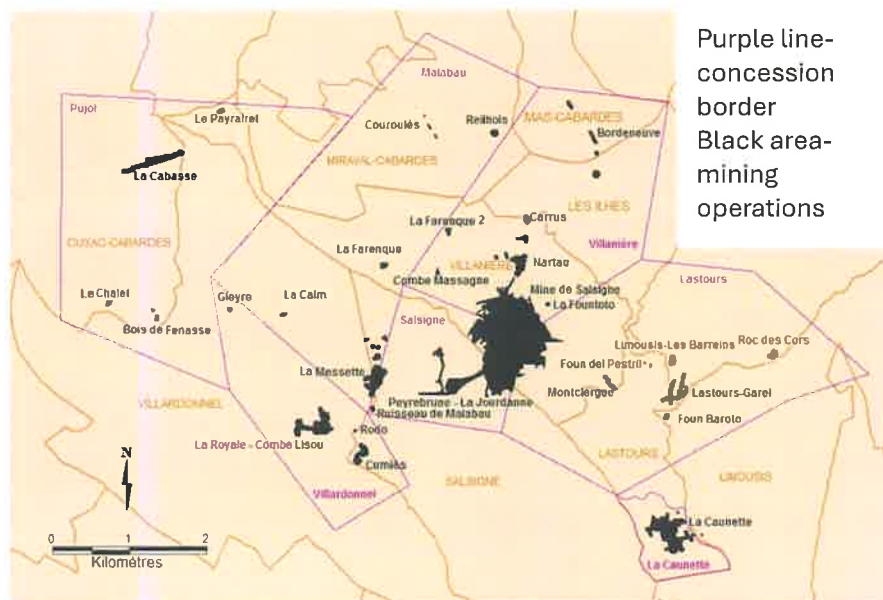


Figure 3: Map of former concessions and associated mining operations in the Salsigne district (VERMEERSCH, 2012; reproduced by kind permission of GÉODERIS)

2.2.3. Consequences of former mining activities on the environment

Provisions set forth by a prefectural decree prohibited the cultivation, sale, and consumption of specific vegetables, snails, thyme, as well as the use of water from wells in the Orbiel Valley, water from Orbiel River and its tributaries, since 1997 due to the elevated levels of arsenic contamination in both soil and irrigation water (DROUHOT et al., 2014). This decree has been renewed ever since on a regular basis. Arsenic is transmitted to the environment of the

Salsigne district either in particulate form (dust), or as dissolved fractions (acid mine drainage) (KHASKA et al., 2018). In October 2018, extreme precipitations lead to a *100-year flood* event, raising concerns all over the valley about the possibility of further contamination and bringing to light the issues created by decades of mining (CALAS et al., 2024). One such example is the *verse de Nartau* where elevated soil levels of arsenic (more than 30000 mg kg⁻¹ on several points) were identified on the surface, with the Grésillou stream situated right below the location (ICF ENVIRONNEMENT, 2007).

Air quality in the Orbiel Valley in 2022 was qualified as good (same as in 2006 and 2020), with PM₁₀ levels in range of EU standards. Source of elements found in PM₁₀ and plant species used for monitoring the pollution in the Valley was assumed to be resuspensions of dust and of anthropogenic materials found at highest levels at locations of waste storage facilities and former industrial mining sites (CALAS et al., 2024). Surface and groundwater content of arsenic (As) down-gradient from the reclaimed ore processing site was higher than the regional background (KHASKA et al., 2015). KHASKA et al. (2018) also reported cycling of As in the hydro-geosystem of that area. The As concentrations measured in the river sediments of the Orbiel valley were of the same order of magnitude as published in the literature for environments strongly impacted by mining or mineral processing activities (DELPLACE, 2022). Background concentrations of arsenic in the soil of Salsigne district were two times higher than median background values for French soil (DROUHOT et al., 2014). In addition, arsenic in soil from remediated and non-remediated zones of former mining activities were even higher, crossing the thresholds for deleterious effects in terrestrial mammals living in or on contaminated soil (DROUHOT et al., 2014). Arsenic in Salsigne's soil was proven to have high capacity for mobilization in the environment, especially under acidic and/or reducing conditions, present in mining locations (BISONE et al., 2016).

Only few studies involved animals for monitoring pollution of the Salsigne district. Two wild rodent species were used to reflect consequences of different remediation actions done in soil. However, arsenic in their organs did not show clear decline among all remediated locations compared to non-remediated locations, as was the case in soil. Body and organ mass reduction in these small mammals were moderate and associated with environmental arsenic concentrations (DROUHOT et al., 2014). Other study involving bees situated near the former mining site of Salsigne revealed impaired cognitive function and physical modifications, which could affect their behaviour (MONTCHANIN et al., 2024). To the best of the author's knowledge, no studies have been conducted on pet animals in this region.

Since 2019, no official human monitoring studies have been done in the Salsigne district regarding arsenic levels. Five years ago, the ARS (*Agence régionale de Santé*) conducted individual clinical and biological monitoring of exposure to arsenic in children under 11 years of age (JO SÉNAT, 2019). Out of 191 children included in the monitoring, 58 (30%) had urine arsenic levels above the reference value for the French population ($10 \mu\text{g g}^{-1}$ creatinine) (ARS, 2019a; ARS, 2019b). After two months, a follow up study was done on 25 children with initially high arsenic values. In five of these children, arsenic levels in urine were still exceeding the reference value ($10 \mu\text{g g}^{-1}$ creatinine), while the remaining 20 children had their arsenic values below reference value (ARS, 2019b). Earlier study done in 1997 on 478 persons (adults and children) from Salsigne district reported 3.8% of inhabitants having arsenic in the urine higher than $15 \mu\text{g g}^{-1}$ creatinine and in five of them level exceeded $20 \mu\text{g g}^{-1}$ creatinine (FRÉRY et al., 1998). Majority of these inhabitants had arsenic levels in the urine below the reference value proposed for adult French population (SAOUDI et al., 2012).

3. MATERIALS AND METHODS

The research included dogs from a control population and an exposed population. The exposed population included dogs living in seven cities within the Orbiel Valley, France, up to 10 km from Salsigne mine. The control population consisted of dogs from nine cities in central France, approx. 420 km from Orbiel Valley, in the range of 20 km from the main sampling site in Francheville. The sampling was conducted over a ten-month period in 2023 for the exposed group, while the control group was sampled over a three-month period in 2023.

3.1. Ethical commitment

Approval for this study was granted by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Zagreb (Klasa: 640-01/23-02/13, Ur.br. 251-61-01/139-23-17) on 20 December 2023. The dog owners provided written informed consent to take part in the study, which included sampling of their dog's hair and nails, together with completing a questionnaire.

3.2. Questionnaire – information collection

Given the potential for a number of factors to affect the concentration of arsenic in hair and nails (ROSENDAHL et al., 2022), a questionnaire was provided for each sampled dog to collect basic data concerning the animal. Dog owners provided name, breed (if applicable), sex, age, and weight (in kilograms, kg), followed by the living conditions and medical history of their dog. They also indicated the length of time their dog had resided in the area, its habits, and the frequency it swam in local rivers with a particular emphasis placed on the Orbiel river and its tributaries within the context of the exposed group. Owners of working dogs, such as hunting dogs indicated details and frequency of the dogs' activity. Furthermore, data were collected regarding the type of food (e.g. dry, or wet) and the specific protein and cereal components within that food. Additionally, the source of the water (tap or well water) was indicated. In addition, dog owners from the control group were queried as to whether their dogs had been in contact with potentially contaminated land, such as old mining districts, and how often. The dog owners from the exposed group were also asked to provide details of their walking habits, specifically whether they walked frequently outside urban areas within the valley as well as if they had been directly exposed to the 2018 floods or their consequences. Lastly, owners indicated if dogs had diagnosed chronic conditions (if any) and the duration of the illness.

3.3. Sample collection

Up to 10 grams of unwashed hair was cut from the neck or shoulder region with stainless steel scissors as closely as possible to the skin, while nails were trimmed with stainless steel nail trimmers. The choice of the shoulder/neck region for sampling was based on the premise that it is the one of the fastest regions to undergo hair regrowth subsequent to clipping (GUNARATNAM and WILKINSON, 1983). To prevent cross-contamination with other dogs' hairs and from a hygienic standpoint, scissors and trimmers were cleaned and disinfected with alcohol between each dog. Not all samples reached the required 10 grams for a variety of reasons. These include the presence of dermatological disorders in the sampling area, as was the case with sample E5 (dog from exposed area), as well as noncompliance or insufficient surface area in younger and smaller dogs. Paired nail and hair samples were placed in the same envelope and stored at room temperature. To prevent possible contamination and loss of material, the envelopes were sealed with masking tape. The names of the animals, their owners, the date of collection, and the location where the samples were obtained were written on the front of the envelopes to ensure traceability together with code number (Figure 4). Thereafter, the envelopes were stored in a cardboard box prior to being transported to Croatia, at the end of September 2023. The protocol for sampling and storage was consistent across all samples in both control and exposed group.



Figure 4: Enveloppe containing a hair sample from exposed group. Name of the dog and the owner is blanked (Photo by Maja Lazarus)

3.3.1. Exposed group

Dogs living in the cities of Miraval-Cabardès, La Tourette-Cabardès, Mas-Cabardès, Lastours, Les Ilhes, Salsigne, Conques-sur-Orbiel, Villalier, Villardonnell, Villanière, Bouilhonnac, Limousis, Trèbes and Villemoustaussou (Figure 5) were included in the exposed group. Dogs were eligible if they were over six months old and had lived in the aforementioned cities for more than three months. In addition, short-haired dogs were also eligible if their owners were in agreement with the temporary cosmetic issue of bare skin on a more or less extensive area of around 20cm. The mayors of the aforementioned cities were contacted via mail or e-mail to inform them about the study as well as the BRGM (*Bureau de Recherches Géologiques et Minières*) and the ARS (*Agence Régionale de Santé*) *Délégation départementale de l'Aude*. Additionally, several veterinarians and dog groomers were contacted via both phone and email in between December and January 2023 to inquire about potential collaboration.

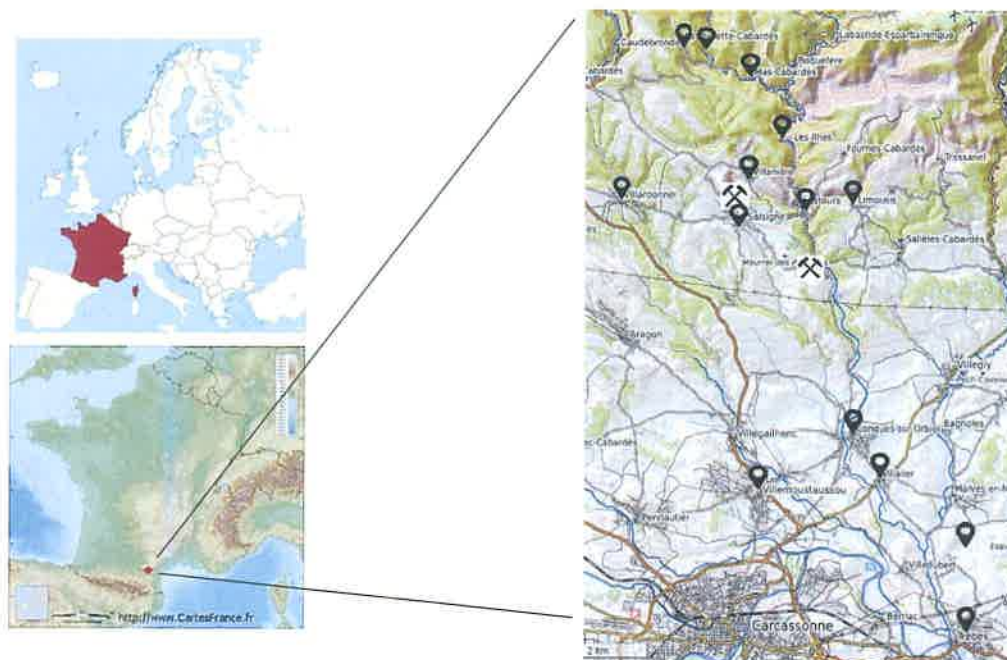


Figure 5: Location of the Orbiel valley and cities where dogs from the exposed group reside (TUBS, 2011; CARTESFRANCE, 2024a; TOPOGRAPHIC-MAP.COM, 2024)

A total of twenty-seven canines (15 females and 12 males) from the exposed area were sampled over a ten-month period, with paired samples of hair and nail collected from eight of these animals (Table 1). Sampling was conducted at a veterinary clinic in Villemoustaussou between February and June 2023 for a total of eight dogs. For an additional eleven samples,

collection was undertaken by a mobile grooming establishment between January and September 2023. The remaining seven samples were collected at the place of residence of the dogs by the author of this Thesis in September 2023.

Table 1: The biometric data of dogs from the exposed group (E) sampled in this study¹

Dog ID	Age (y)	Sex	Weight (kg)	Breed	Date	Place of residence	Type of sample
E1	13	F	7 to 8	X Pinscher	08/09/2023	Conques-sur-Orbiel	Hair
E2	4.5	F	4.5	Spitz	08/09/2023	Conques-sur-Orbiel	Hair
E3	4	M	10	Spitz	08/09/2023	Conques-sur-Orbiel	Hair
E4	3	M	9	Spitz	08/09/2023	Conques-sur-Orbiel	Hair
E5	8	F	18-20	French Setter	08/09/2023	Conques-sur-Orbiel	Hair
E6	2.5	M	>15	Cocker Spaniel	08/09/2023	Miraval-Cabardès	Hair
E7	5.5	M	14	<i>Petit basset griffon vendéen</i>	08/09/2023	Miraval-Cabardès	Hair
E8	7	F	9	CKC	17/03/2023	Conques-sur-Orbiel	Hair + Nail
E9	6 to 7	M	12.8	Daschund X York	03/02/2023	Villardonnel	Hair + Nail
E10	7	F	16	Shiba Inu	04/01/2023	Conques-sur-Orbiel	Hair
E11	6	M	18	Shiba Inu	04/01/2023	Conques-sur-Orbiel	Hair
E12	3	F	12.5	Shiba Inu	03/02/2023	Villemoustaussou	Hair
E13	2	M	12	Brittany Spaniel	07/09/2023	Conques-sur-Orbiel	Hair + Nail
E14	6	M	10	Brittany Spaniel	07/09/2023	Conques-sur-Orbiel	Hair + Nail
E15	5	F	15	X Shepherd	07/09/2023	Conques-sur-Orbiel	Hair + Nail
E16	10	F	22	Border Collie	07/09/2023	Conques-sur-Orbiel	Hair + Nail
E17	9	M	24	Border X Staff	07/09/2023	Conques-sur-Orbiel	Hair + Nail
E18	0.66	M	30	American Bully	07/09/2023	Conques-sur-Orbiel	Hair + Nail
E19	12	M	38	Golden Retriever	02/23	Salsigne	Hair
E20	5.5	F	40	Labrador	16/06/2023	Conques-sur-Orbiel	Hair
E21	9	F	9.8	Long haired ratter	06/06/2023	Villemoustaussou	Hair
E22	14	F	17	X	12/06/2023	Villemoustaussou	Hair
E23	8	F	14	Épagneul breton	12/05/2023	Conques-sur-Orbiel	Hair
E24	6.5	F	5	Yorkshire terrier	12/06/2023	Villanière	Hair
E25	6	M	66	X shepherd	28/04/2023	Villemoustaussou	Hair
E26	4	F	6.5	X wire haired daschund	15/06/2023	Limousis	Hair
E27	3	F	15	Border collie	15/06/2023	Limousis	Hair

¹Age in years (estimated for E9); M-male, F-female; weight in kg (estimated for E1, E5 and E6), X- crossbreed; CKC- Cavalier King Charles

3.3.2. Control group

Dogs living within 20 km north of the main control sampling site (Francheville, near Lyon, France) were included, provided they were over six months old and had lived there for more than three months.

There is a lack of publicly available data regarding the concentration of arsenic in the soil of the Rhône department. However, one study indicated that arsenic soil levels from the area where the sampled dogs reside were estimated to be between 15 and 25 mg kg⁻¹ (LOISEAU et al., 2021). This concentration is not the lowest recorded in France, but it falls within the range of normal values. The estimated mean soil arsenic concentration in France is 19.47 mg kg⁻¹. The sampled canines were not residing in proximity to identified deposits or contaminated soils, as indicated by the ex-BASOL (*BAse de données des sites et SOLs pollués*) inventory of polluted soils (GÉORISQUES: MINISTÈRE DE LA TRANSITION ÉCOLOGIQUE ET DE LA COHÉSION DES TERRITOIRES, 2024) and the CASIAS (*Carte des Anciens Sites Industriels et Activités de Services*) map of previous industrial areas (GÉORISQUES: MINISTÈRE DE LA TRANSITION ÉCOLOGIQUE ET DE LA COHÉSION DES TERRITOIRES, 2021).

Dogs from control group inhabited nine cities including the 5th arrondissement of Lyon, Craponne, Saint-Genis-lès-Ollières, Tassin-la-Demi-Lune, Dommartin, Sainte-Foy-lès-Lyon, Chaponost and Chasselay (Figure 6).

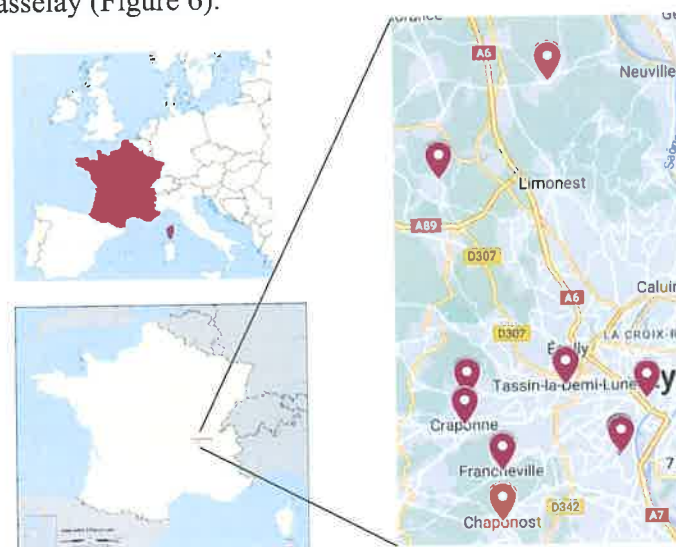


Figure 6: Location of Francheville and cities where dogs from the control group reside (France in TUBS (2011); CARTESFRANCE (2024b), GOOGLEMYMAPS (2024))

A total of twenty-two canines (10 females and 12 males) from the control area were sampled over a three-month period of 2023, with paired samples of hair and nail collected from six of these animals (Table). Sampling for 17 dogs was conducted by the author at a dog grooming establishment in Francheville on the 17th, 19th and 27th of July 2023. One sample was obtained in June 2023 by the dog's owner in accordance with the instructions provided on an enclosed instructions sheet, while the remaining four samples were collected by the author at the place of residence of the dogs in between July and September 2023.

Table 2: The biometric data of dogs from the control group (C) sampled in this study¹

Dog ID	Age (y)	Sex	Weight (kg)	Breed	Date	Place of residence	Sample
C1	0.75	M	15.5	Labrador X Poodle	19/07/2023	Tassin-la-Demi-Lune	Hair
C2	4	F	17	Cocker	19/07/2023	Sainte-Foy-lès-Lyon	Hair
C3	8	F	5	Lhasa apso	19/07/2023	Dommartin	Hair
C4	6	M	5.5	Yorkshire terrier	19/07/2023	Sainte-Foy-lès-Lyon	Hair
C5	9	F	-	Shih Tzu	19/07/2023	Chaponost	Hair
C6	2	F	4.5	Westie	19/07/2023	Tassin-la-Demi-Lune	Hair
C7	11	F	4.5	Yorkshire terrier	19/07/2023	Sainte-Foy-lès-Lyon	Hair
C8	8	F	-	Golden retriever	19/07/2023	Chaponost	Hair
C9	11	M	10	Golden X Spaniel	17/07/2023	Craponne	Hair
C10	2	M	8	Spitz	17/07/2023	Francheville	Hair
C11	4	M	8	Spitz	17/07/2023	Francheville	Hair
C12	16	M	3	<i>Papillon</i>	17/07/2023	Lyon 69005	Hair + nail
C13	5.6	M	31	Australian Shepherd dog	30/07/2023	Chasselay	Hair
C14	5	F	50	BMD	28/07/2023	Craponne	Hair
C15	10	M	20	Cocker	28/07/2023	Tassin-la-Demi-Lune	Hair + nail
C16	12	F	25	Springer	28/07/2023	Sainte-Foy-lès-Lyon	Hair
C17	7	F	6.5	Shih Tzu	28/07/2023	Francheville	Hair + nail
C18	7	M	7.5	Havanese	28/07/2023	Saint-Genis-les-Ollières	Hair + nail
C19	8	M	21	Labrador Retriever	16/07/2023	Francheville	Hair + nail
C20	5	M	11	X Puli	17/07/2023	Francheville	Hair + nail
C21	10	M	25	Australian shepherd dog	10/06/2023	Francheville	Hair
C22	8	F	25	Golden retriever x PMD	21/09/2023	Francheville	Hair

¹Age in years; M-male, F-female; weight in kg (- no data); X- crossbreed; BMD- Bernese Mountain Dog; PMD- Pyrenean Mountain Dog

3.4. Equipment and chemicals

- Lab Analytical Balance MS303S, Mettler Toledo, Columbus Ohio, USA
- Sub-boiling distillation system SubPUR/DuoPUR for HNO₃ purification, Milestone, Italy
- Vortex Genius 3, IKA, Germany
- Water Purification System Smart2Pure 6 UV/UF, Thermo Scientific, Massachusetts, USA
- Drying oven Heratherm™ OHG60, Thermo Fisher Scientific, Massachusetts, USA
- Inductively coupled plasma mass spectrometer Agilent 8900 ICP-MS/MS, Agilent Technologies, Japan
- Microwave digestion system UltraCLAVE IV, Milestone, Italy
- Arsenic solution (As), $\rho = 1001$ mg/L, Inorganic Ventures, Christiansburg, Virginia, USA
- Ar, plasma gas, purity >99,999%, UTP d.o.o., Zagreb, Croatia - SOL Group, Italy
- He, collision gas, purity >99,9999%, UTP d.o.o., Zagreb, Croatia - SOL Group, Italy
- Reference material Human hair IAEA-086, Austria
- Certified reference material No.13 Human hair, National Institute for Environmental Studies, Japan
- Acetone for gas chromatography MS SupraSolv®, Merck, Germany
- Purified conc. nitric acid (HNO₃) (duoPUR/subPUR, Milestone, Italy) – originates from conc. nitric acid (HNO₃, 65%, p.a.), Merck, Germany
- Ultrapure water: electric conductivity 0.055 μ S/cm (at 25°C; 18 M Ω cm), Smart2Pure 6 UV/UF system

3.5. Sample washing and digestion

In order to eliminate potential sources of external contamination with arsenic, such as dust, water, and possible hygienic products (SKRÖDER et al., 2017), the hair and nail samples were washed. Approximately 0.2 g of hair sample was weighted in a 50 mL tube where 20 mL of ultrapure water was added and subjected to a 10-minute vortexing process as pre-washing step. Subsequently, a five-step washing procedure (acetone-water-water-water-acetone; RYABUKHIN, 1976) was conducted in accordance with the recommendations set forth by the International Atomic Energy Agency (IAEA). The ultrapure water and acetone for gas chromatography were utilised. Each phase comprised a 10-minute vortex with 20 mL of solvent

(as detailed in LAZARUS et al., 2020). Due to the smaller size of the nail samples and the correspondingly smaller surface area, it was necessary to adapt the amount of chemicals used in washing steps from 20 mL to 10 mL. Subsequent to washing stages, both the hair and nails were dried for 24 hours at 40 °C. Tubes were covered with thin cellulose sealed with an elastic band in order to prevent contamination and allow the evaporation of water during the drying process. Samples were then transferred to Teflon tubes and weighed. Subsequently, 2 ml of ultrapure water and 2 ml of purified nitric acid were added to the tubes. The tubes were then closed with a Teflon cap, placed on a rack, and digested in an UltraCLAVE IV microwave digestion system following a five-step process presented in the table 3. Following cooling, samples were all adjusted to 5 g with ultrapure water.

Table 3: Parameters for each step of the digestion process

Step	Time (min)	Energy (W)	Temperature (°C)	Pressure (bar)
1	3.30	700	70	100
2	15.00	1000	180	100
3	10.00	1000	260	140
4	30.00	1000	260	140
5	40.00	0	30	20

The certified reference material (CRM) No.13 Human hair and two blank samples (2 ml of ultrapure water and 2 ml of purified nitric acid) followed the exact procedure in parallel to hair and nail samples to control the quality of digestion method.

3.6. Analyses of arsenic levels in the samples

Prior analysis of arsenic, hair and nail samples, together with reference materials were diluted with ultrapure water thereby ensuring that all samples were within the calibration range of standards for arsenic. Arsenic was quantified by inductively coupled plasma mass spectrometry (ICP-MS), in accordance with the accepted procedure detailed in VIHNANEK LAZARUS et al. (2013) and LAZARUS et al. (2020). Inductively coupled plasma mass spectrometry offers a number of advantages, including the capacity to analyse multiple elements concurrently, a relatively short analysis time and a straightforward sample preparation process. The ICP-MS apparatus employs an argon (Ar) plasma, to which the digested samples are introduced. This process causes the samples to split into individual atoms, which lose electrons and become positive charged ions. The ions are then extracted, separated according

to their mass-charge ratio (m/z), and quantified using a mass spectrometer (WILSCHEFSKI and BAXTER, 2019).

Limit of detection (LOD) calculated as average concentration of the blank samples plus three times the standard deviation of the blank concentration ($n=10$), was $1.84 \mu\text{g As kg}^{-1}$ for hair samples, and $2.70 \mu\text{g As kg}^{-1}$ for nail samples. Limit of quantification (LOQ), calculated as average concentration of the blanks plus ten times the standard deviation, was $3.32 \mu\text{g As kg}^{-1}$ for hair samples, and $4.86 \mu\text{g As kg}^{-1}$ for nail samples.

Quality assurance of the analytical method was conducted by simultaneously analysing the hair and nail samples alongside the CRM of matching matrix (hair). Information value for arsenic in CRM No. 13 Human hair is $100 \mu\text{g kg}^{-1}$, while the measured value was 101 (range 96-106) $\mu\text{g kg}^{-1}$.

3.7. Statistical analyses

Normality of distribution for arsenic data was tested using Shapiro-Wilk's test, which showed non-normal distribution. Descriptive statistics were thus presented with mean, standard deviation, median and range in tables, and additionally with first (Q1, 25th percentile) and third quartile (Q3, 75th percentile) in figures. The Box Cox transformation was applied to hair data and quantile transformation was applied to nail data to achieve normality. Differences in hair arsenic level between control and exposed group were tested using the Welch's t-test, as the assumption of equal variances (homoscedasticity) was not met (Levene's test). Means of nail arsenic levels between the same two groups were tested using the Student's t-test, because the variances were equal. Differences between two sexes and swimming habits of dogs were tested using the Student's t-test for both arsenic hair and nail data. The Pearson correlation coefficient (r) and simple linear regression were employed to examine the presence of linear associations between numerical variables (arsenic in hair/nails vs. age, body mass, residence period, and arsenic in hair vs. arsenic in nail). Results of linear regression include goodness of fit (adjusted R-squared, R^2), f with degrees of freedom ($f(df)$) and p-value. Associations were interpreted according to HINKLE et al. (2003), where $0.3 < r \leq 0.5$ indicated low correlation, $0.5 < r \leq 0.7$ moderate correlation, $0.7 < r \leq 0.9$ high correlation, and $r > 0.9$ very high correlation.

The level of significance was set at $\alpha=0.05$. Data were analyzed using JMP 17.0.0 Pro (SAS Institute Inc, Cary, North Carolina).

4. RESULTS

4.1. Biometric data, residence period and swimming habits of dogs included in this study

A total of 49 dogs were sampled for this study. Of these, 22 were assigned to the control group based on residence in the Francheville area, near Lyon, and 27 to the exposed group based on residence in the Salsigne district. Biometric data obtained from the questionnaire included sex, age, body mass, as well as residence period in the Orbiel Valley and swimming habits of dogs from the exposed group. Mean, standard deviation of the mean, median, and range of aforementioned biometric data are summarized in Table 4. Study included 24 male (12 in both control and exposed groups) and 25 female (10 in control and 15 in exposed group) dogs. Two dogs from the control group did not have their body mass indicated. The youngest dog was 7 months old, and the oldest was 16 years old. Average age and body mass of dogs were similar in both groups. Dogs from the exposed group lived in the Salsigne area for 6 years on average.

Table 4: Summary statistics for biometric data of 49 dogs (*Canis lupus familiaris*) from control (N=22) and exposed group (N=27) and residence period of dogs from exposed group¹

		All	Control group	Exposed group
Sex	N	24 M, 25 F	12 M, 10 F	12 M, 15 F
Age (years)	Mean±SD	6.89±3.55	7.24±3.65	6.55±3.49
	Med, range	7.0, 0.66-16.0	7.5, 0.75-16.0	6.0, 0.66-14.0
Mass (kg)	Mean±SD	18.6±13.2	18.5±12.6	18.7±13.9
	Med, range	15.0, 3.0-66.0	18.5, 3.0-50.0	15.0, 4.5-66.0
Residence period (years)	Mean±SD			6.0±3.57
	Med, range			6.0, 0.3-14.0

¹N= number of dogs; M= Males, F= Females; SD= Standard Deviation; Med= Median

Number of dogs with and without the habit of regular swimming in the rivers and streams of the Salsigne district is presented in Figure 7.

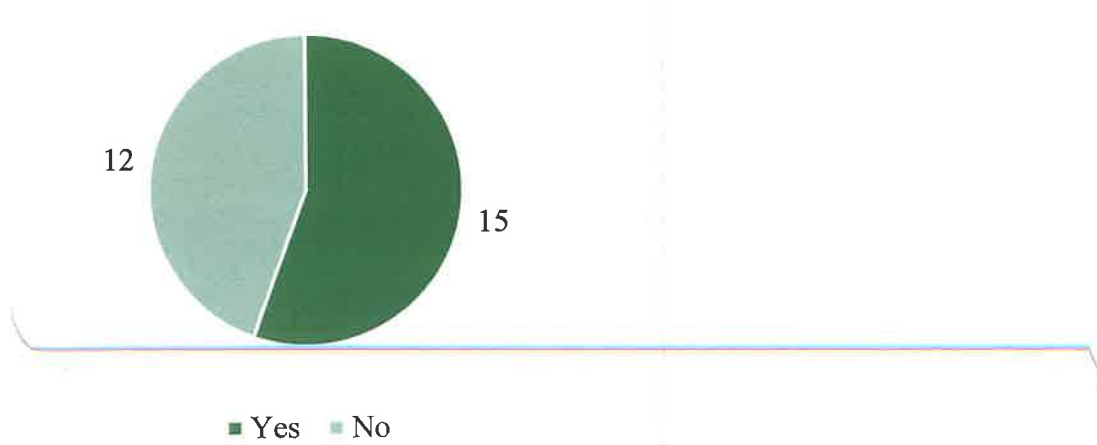


Figure 7: Pie chart of swimming habits and number of respective dogs from exposed group (N=27). Yes indicates dogs (N=15) that regularly swam in the local rivers of Salsigne district. No indicates dogs (N=12) that had no habit of swimming in the local rivers.

4.2. Differences in arsenic hair and nail levels between dogs from control and exposed group

Arsenic levels were quantified in all samples of hair and nail, i.e., they were above the limit of detection of used method. Mean and standard deviation of mean, median and range calculated for arsenic levels in hair of dogs sampled in this study are presented in Table 5. Testing mean arsenic levels in hair between control and exposed group revealed statistically significant difference (Welch's t-test, $t(46)=2.26$, $p=0.029$). Dogs from control group had significantly lower hair arsenic compared to dogs from exposed group (Table 5). Differences in arsenic hair levels between two groups are graphically presented in Figure 8.

Table 5: Summary statistics for arsenic level ($\mu\text{g kg}^{-1}$) in hair of 49 dogs and results of testing differences (Welch's t-test) between control (N=22) and exposed group (N=27)¹.

	All	Control group	Exposed group	Difference C vs. E p-value
N	49	22	27	
Mean±SD	160±43	70.9±99.7	232±382	0.029
Med, Range	58.1, 17.8-1759	48.3, 21.0-506	86.8, 17.8-1759	

¹N= number of dogs; SD= Standard Deviation; Med= Median

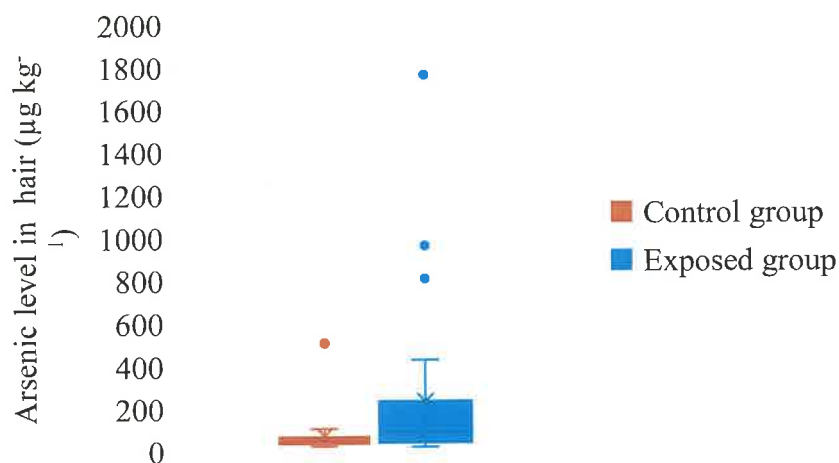


Figure 8: Arsenic level ($\mu\text{g kg}^{-1}$) in hair of 49 dogs (22 from control group; 27 from exposed group). Boxes denote first and third quartile with central line as median, cross denotes mean, whiskers denote non-outlier range, circles denote outliers.

Mean and standard deviation of mean, median and range calculated for arsenic levels in nails of dogs sampled in this study are presented in Table 6. Arsenic level ranged higher in the exposed group, but the statistical difference between exposed and control group was not significant (Student's t-test, $t(12)=1.29$, $p=0.222$). Differences in arsenic nail levels between two groups are graphically presented in Figure 9.

Table 6: Summary statistics for arsenic level ($\mu\text{g kg}^{-1}$) in nails of 14 dogs and results of testing differences (Student's t-test) between control (N=6) and exposed group (N=8)¹

	Control group	Exposed group	Difference C vs. E p-value
N	6	8	
Mean±SD	137±87.0	722±815	0.222
Med, Range	125, 30.4-285	175, 84.4-1952	

¹N= number of dogs; SD= Standard Deviation; Med= Median

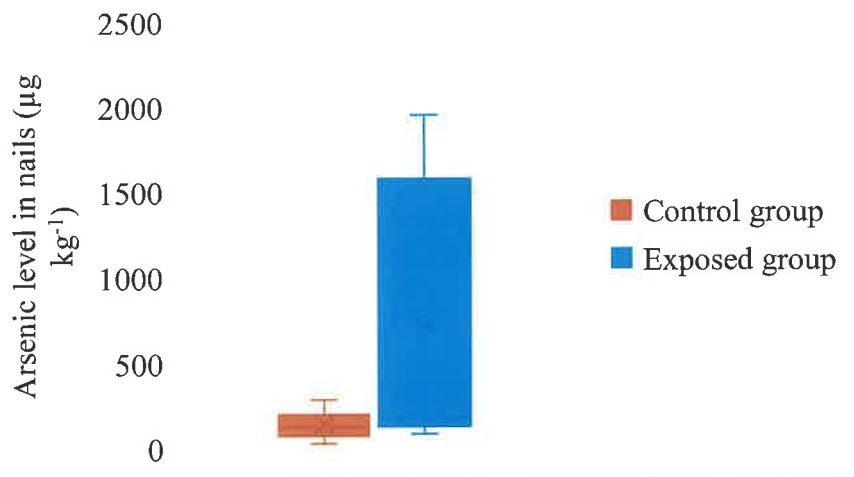


Figure 9: Arsenic level ($\mu\text{g kg}^{-1}$) in nails of 14 dogs (6 from control group; 8 from exposed group). Boxes denote first and third quartile with central line as median, cross denotes mean, whiskers denote range.

4.3. Arsenic levels in hair and nails of dogs in relation to sex

This study included 24 male and 25 female dogs. Mean, median, interquartile range, range without outliers and outliers calculated for arsenic levels in hair of dogs sampled in this study are presented categorized by sex in Figure 10. Student's t-test revealed absence of statistically significant difference between hair arsenic level in males and females ($t(47)=-0.342$, $p=0.734$), although maximum values were noted in the group of male dogs.

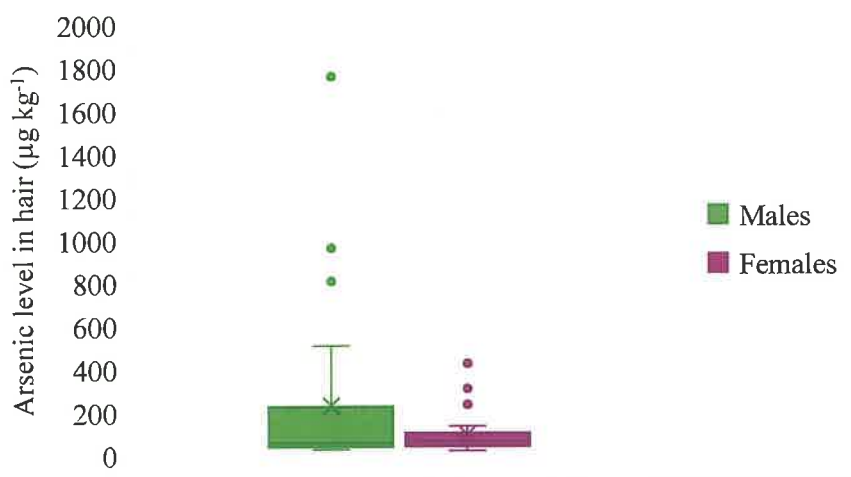


Figure 10: Arsenic level ($\mu\text{g kg}^{-1}$) in hair of 49 dogs (24 males; 25 females). Boxes denote first and third quartile with central line as median, cross denotes mean, whiskers denote non-outlier range, circles denote outliers.

Nails were sampled from 14 dogs in total. Mean, median, interquartile range, range without outliers and outliers calculated for arsenic levels in nails of dogs sampled in this study are presented categorized by sex in Figure 11. Student's t-test revealed absence of statistically significant difference between hair arsenic level in males and females ($t(12)=0.357$, $p=0.727$).

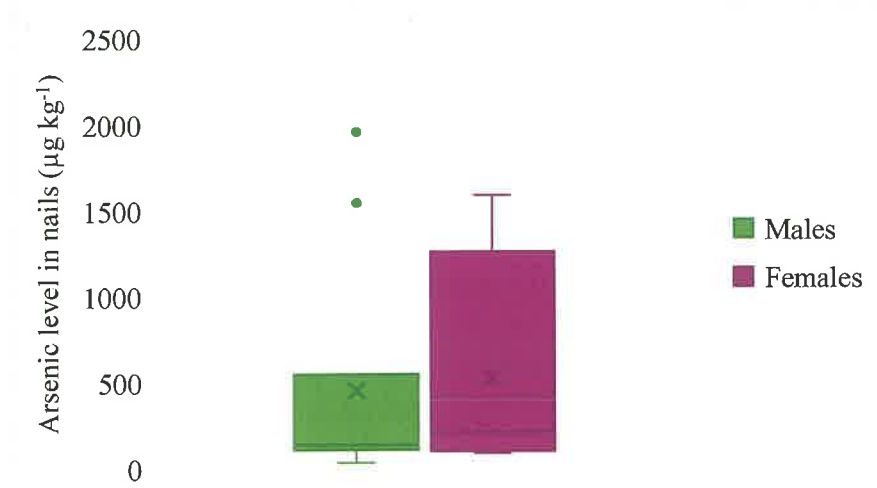


Figure 11: Arsenic level ($\mu\text{g kg}^{-1}$) in nails of 14 dogs (10 males; 4 females). Boxes denote first and third quartile with central line as median, cross denotes mean, whiskers denote non-outlier range, circles denote outliers.

4.2. Arsenic level in hair and nails of dogs in relation to age

Arsenic levels in hair of 49 dogs in relation to age of dogs are presented in Figure 12. Testing association between arsenic hair level and age using the simple linear regression revealed negative non-significant relation ($R^2=0.04$, $f(1,43)=2.61$, $p=0.113$).

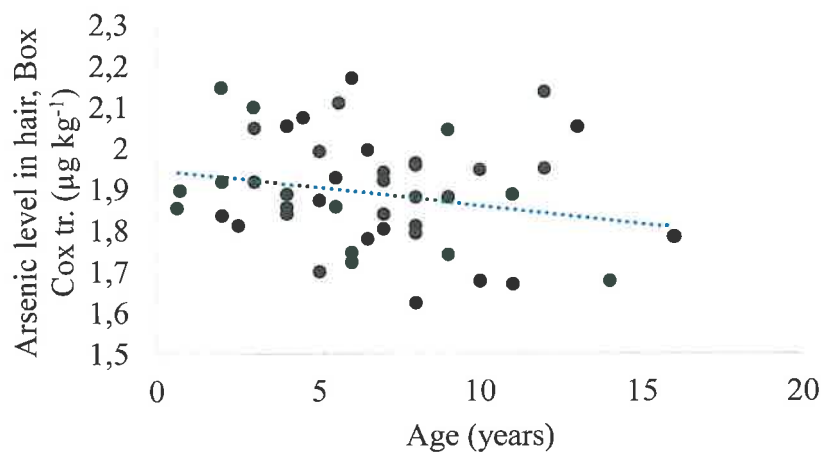


Figure 12: Arsenic level (Box Cox transformed) in hair ($\mu\text{g kg}^{-1}$) of 49 dogs in relation to age. Blue dotted line denotes negative non-significant linear association ($p=0.113$)

Arsenic levels in nails of 14 dogs in relation to age of dogs are presented in Figure 13. Testing association between arsenic nail level and age using the simple linear regression revealed negative non-significant relation ($R^2=0.20$, $f(1,12)=4.27$, $p=0.061$).

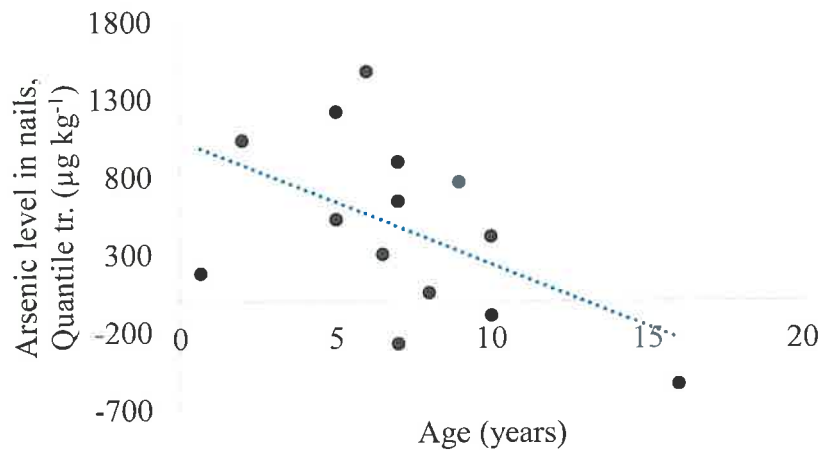


Figure 13: Arsenic level (Quantile transformed) in nails ($\mu\text{g kg}^{-1}$) of 14 dogs in relation to age. Blue dotted line denotes negative non-significant linear association ($p=0.061$).

4.4. Arsenic level in hair and nails of dogs in relation to their body mass

Arsenic levels in hair of 47 dogs in relation to their body mass are presented in Figure 14. Two dogs were excluded from the control group as their body mass was not known. Testing association between arsenic hair level and body mass using the simple linear regression revealed negative non-significant relation ($R^2=0.03$, $f(1,35)=2.01$, $p=0.165$).

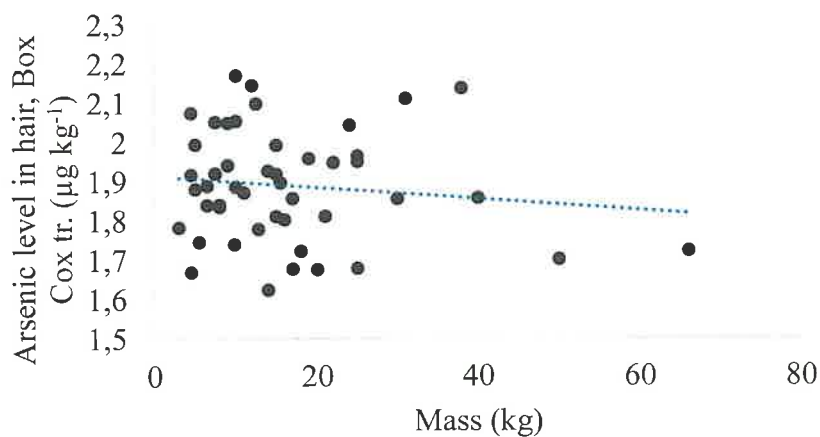


Figure 14: Arsenic level (Box Cox transformed) in hair ($\mu\text{g kg}^{-1}$) of 47 dogs in relation to their body mass. Blue dotted line denotes negative non-significant linear association ($p=0.165$).

Arsenic levels in nails of 14 dogs in relation to their body mass are presented in Figure 15. Testing association between arsenic nail level and body mass using the simple linear regression revealed negative non-significant relation ($R^2=-0.11$, $f(1,9)=0.02$, $p=0.882$).

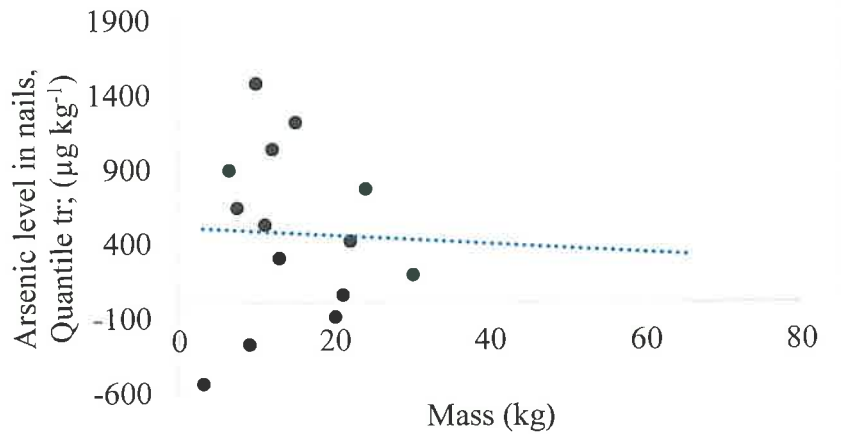


Figure 15: Arsenic level (Quantile transformed) in nails ($\mu\text{g kg}^{-1}$) of 14 dogs in relation to their body mass. Blue dotted line denotes negative non-significant linear association ($p=0.882$)

4.5. Arsenic level in hair and nails of dogs from exposed group in relation to residence period in the Salsigne district

Arsenic levels in hair of 27 dogs from exposed group in relation to years that dogs reside in the Salsigne district are presented in Figure 16. Testing association between arsenic hair level and residence period using the simple linear regression revealed negative non-significant relation ($R^2=-0.01$, $f(1,22)=0.69$, $p=0.414$).

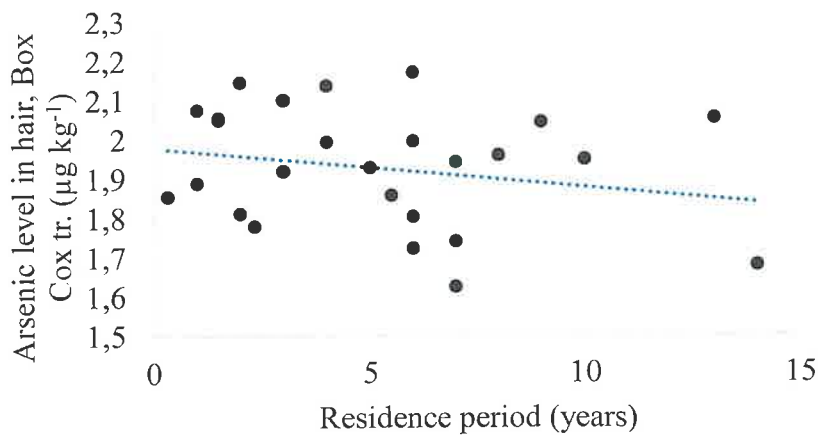


Figure 16: Arsenic level (Box Cox transformed) in hair ($\mu\text{g kg}^{-1}$) of 27 dogs in relation to

residence period in exposed area of Salsigne district. Blue dotted line denotes negative non-significant linear association ($p=0.414$).

Arsenic levels in nails of 8 dogs from exposed group in relation to years that dogs reside in the Salsigne district are presented in Figure 17. Testing association between arsenic nail level and residence period using the simple linear regression revealed negative non-significant relation ($R^2=-0.17$, $f(1,6)=0.004$, $p=0.954$).

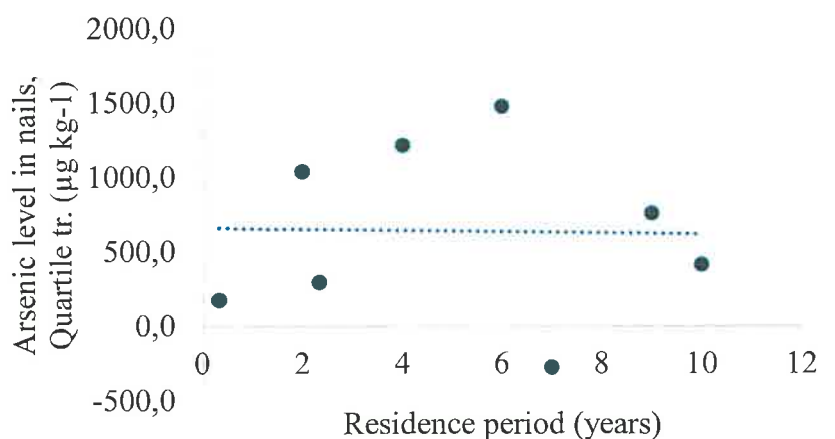


Figure 17: Arsenic level (Quantile transformed) in nails ($\mu\text{g kg}^{-1}$) of 8 dogs in relation to residence period in exposed area of Salsigne district. Blue dotted line denotes negative non-significant linear association ($p=0.954$).

4.6. Arsenic level in hair and nails of dogs from exposed group in relation to their swimming habits in rivers of the Salsigne district

Mean, median, interquartile range, range without outliers and outliers calculated for arsenic levels in hair of 27 dogs are presented in Figure 18 categorized by their habit of swimming in the local rivers of the Salsigne district (15 dogs regularly swim in the local rivers, 12 do not swim in the local rivers). Student's t-test revealed absence of statistically significant difference between hair arsenic level in dogs that have the habit of swimming the local rivers compared to the ones that do not have that habit ($t(25)=-0.646$, $p=0.524$), although higher values were noted in the group of dogs that swim in the rivers of the Salsigne district.

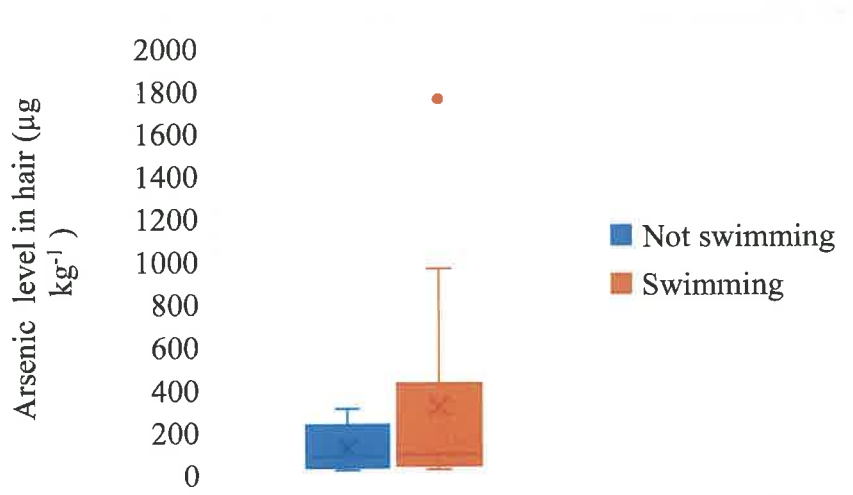


Figure 18: Arsenic level ($\mu\text{g kg}^{-1}$) in hair of 27 dogs from exposed group. Boxes denote first and third quartile with central line as median, cross denotes mean, whiskers denote non-outlier range, circles denote outliers.

Mean, median, interquartile range, range without outliers and outliers calculated for arsenic levels in nail of 8 dogs are presented in Figure 19 categorized by their habit of swimming in the local rivers of the Salsigne district (6 dogs regularly swim in the local rivers, 2 do not swim in the local rivers). Student's t-test revealed absence of statistically significant difference between nail arsenic level in dogs that have the habit of swimming the local rivers compared to the ones that do not have that habit ($t(6)=-2.14$, $p=0.076$), although higher values were noted in the group of dogs that swim in the rivers of the Salsigne district.

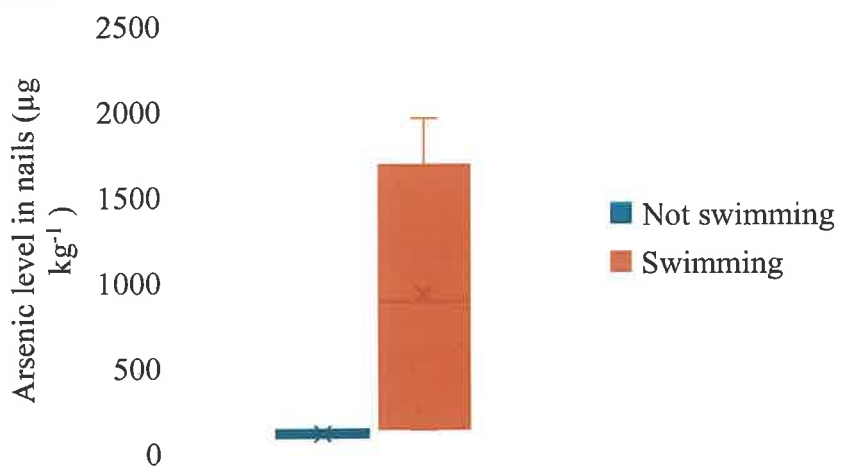


Figure 19: Arsenic level ($\mu\text{g kg}^{-1}$) in nails of 8 dogs from exposed group. Boxes denote first and third quartile with central line as median, cross denotes mean, whiskers denote range.

4.7. Arsenic level in hair in relation to arsenic level in nails of studied dogs

The association between hair and nail arsenic levels in 14 dogs (6 from control group; 8 from exposed group) is represented in Figure 20. The simple linear regression revealed significant positive association between arsenic level in these two matrices ($R^2=0.50$, $f(1,12)=14.2$, $p=0.003$). Pearson coefficient of correlation ($r=0.736$) indicated high correlation.

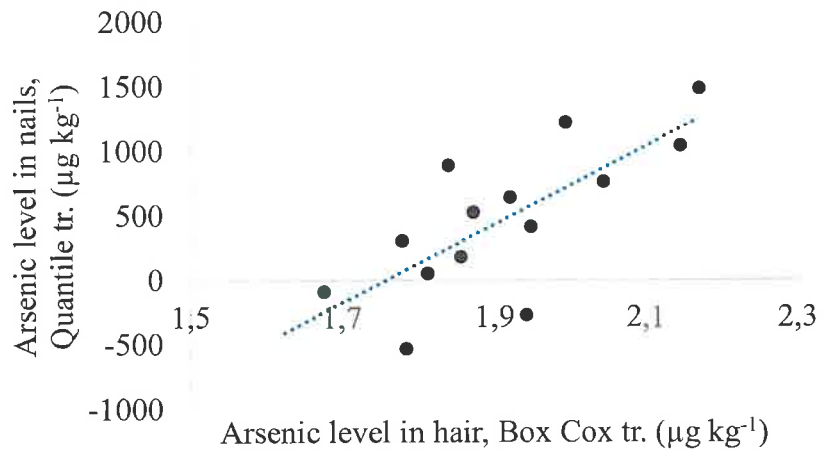


Figure 20: Arsenic level (Box Cox transformed) in hair ($\mu\text{g kg}^{-1}$) in relation to arsenic level (Quantile transformed) in nails ($\mu\text{g kg}^{-1}$) of 14 dogs. Blue dotted line denotes positive significant linear association described by function: $\text{As nail (Q tr)} = -5334 + 3035 \times \text{As hair (Box-Cox tr)}$; ($p=0.003$).

5. DISCUSSION

In this study, we showed that arsenic exposure in dogs from the Salsigne district, a former mining site, is higher than that in dogs from the control area, as reflected by arsenic levels in hair but not in nails. Interestingly, we found a high correlation of arsenic levels between these two matrices when sampled in the same dogs. Additionally, the biological characteristics and some studied habits of the animals did not seem to influence arsenic levels.

Arsenic can enter the body through inhalation, ingestion, and dermal contact with contaminated air, water, food and soil (ATSDR, 2007; TORRES-WONG, 2018; PROUST and PICOT, 2019). Canine exposure to arsenic can result from a number of different sources, including but not limited to industry, pesticides, food, contaminated water, etc. Airborne exposure may result from the combustion of fossil fuels, such as coal, and from emissions released by various industrial processes, including smelters (EISLER, 1988; MOLÉNAT and HOLEMAN, 2000; PROUST and PICOT, 2019), arsenic trioxide production facilities, the wood industry, and semiconductor manufacturing (PROUST and PICOT, 2019). Additionally, the use of herbicide sprays has been identified as a source of airborne arsenic (EISLER, 1988; PROUST and PICOT, 2019). The Orbiel valley is an abundant and ancient viticulture area, which may have suffered from the use of several arsenic pesticides, especially sodium arsenite, which was banned in 2001 (JOURNAL OFFICIEL DE LA RÉPUBLIQUE FRANÇAISE, 2001). Since air quality in the Salsigne district, former mining site chosen as exposed area in this study, was assessed in 2022 as good and in range of EU standards (CALAS et al., 2024), we could not expect air as notable source of exposure for dogs in this area. The presence of arsenic in water may be attributed to natural mineralization processes or the result of anthropogenic activities, such as alterations to the bedrock caused by major public works or mining operations (smelter wastes, mine tailings runoff) (EISLER, 1988; ANTONI and JAMET, 2021). Drinking/tap water for inhabitants of the Salsigne district comes from a source situated northeast of the valley. Regular measurements of tap water during 2024 revealed arsenic levels below the recommended limit in drinking-water of $10 \mu\text{g L}^{-1}$ (WHO, 2022) in Conques-sur-Orbiel ($5\text{-}6 \mu\text{g As L}^{-1}$; ARS, 2024). Most of the dogs from exposed group drank only tap water, with the exceptions of dogs E1, E2, E3 and E4 who occasionally drank well water. Therefore, we find a source of arsenic exposure other than water as responsible for the higher arsenic levels in the hair of dogs from the exposed group compared to control group. As previously stated in this thesis (section 2.1.4.), contaminated water and food could represent significant sources of exposure to arsenic, particularly when food contains rice (inorganic

arsenic) and seafood (organic arsenic). However, arsenic found in hair is in its inorganic form, so diet high in seafood is not expected to influence hair arsenic levels (ROSENDAHL et al., 2020). According to data reported by dog owners, all but two dogs regularly eat commercial dry dog food, often produced with rice (COWELL et al., 2000), which contains inorganic arsenic in variable amounts, depending on the food producer.

Arsenic levels in human hair were shown as a useful screening test to detect chronic arsenic poisoning, as long as special care was taken during sample preparation to avoid and clear external contamination (HINDMARSH, 2002; ATSDR, 2007; KATZ, 2019). KURTIO et al. (1998) found that the arsenic levels in hair reliably predict past and chronic exposure. He also reported that arsenic hair levels correlated with urine levels, while KATZ (2019) reported a positive correlation between levels of arsenic in human hair and in drinking water as well as in blood and/or urine. Additionally, hair exhibited the second highest levels of arsenic in the body followed by nails in a chronically exposed human population (OLGUÍN et al., 1983). Consequently, hair can be considered a reliable biomarker for the evaluation of arsenic exposure. Since dogs share their environment with humans, and a positive association between arsenic levels in the urine of dogs and humans in the same household was confirmed (CRAUN et al., 2020), the results of this study may also be important for human monitoring in the Salsigne area.

Dogs from the exposed group had a higher mean level of arsenic in the hair than dogs from the control group. The highest hair value in the control group ($506 \mu\text{g kg}^{-1}$, C13) was seven times higher than the respective mean of the control group. According to data given by the owner, this dog was spending the summer holidays in an old mining region in France (Lozère department) and swam in the Tarn River, which could influence the higher exposure to environmental arsenic than in other dogs from the control group. The arsenic level in hair ($1759 \mu\text{g kg}^{-1}$, E14) of one dog from the exposed group exceeded $1000 \mu\text{g kg}^{-1}$, the threshold considered normal for human hair and nails (WHO, 1983; ATSDR, 2007).

Table 7 and Table 8 listed arsenic results from this study along with the literature data in hair and nails, respectively, to enable comparison and discussion of measured results. The mean arsenic level measured in French dogs from the exposed group (this study) was 2-3 times higher than the mean in healthy pet dogs living in urban areas in Slovakia (KOZAK et al., 2002). However, urban dogs from Slovakia (KOZAK et al., 2002) and Australia (JAFARI, 2014) had comparable arsenic hair levels to dogs from French control group.

Table 7: Summary of literature data reported for arsenic level ($\mu\text{g kg}^{-1}$) in hair of dogs

Location	Groups		As concentration	Reference
France	Exposed (Salsigne old mining district)	Mean \pm SD	232 \pm 382	This study
		Median	86.8	
		Range	17.8-1759	
	Control	Mean \pm SD	70.9 \pm 99.7	
		Median	48.3	
		Range	21.0-506	
Laboratory exposure (sodium arsenite in diet)	Low dosage	Mean initial	470	NEIGER and OSWEILER, 1992
		Mean final	5980	
	Medium dosage	Mean initial	420	
		Mean final	24470	
	High dosage	Mean initial	400	
		Mean final	24470	
	Control	Mean initial	180	
		Mean final	120	
Slovakia (urban area)	Bratislava	Mean	111	KOZAK et al., 2002
	Kosice	Mean	79.8	
Argentina	Exposed (Los Alamos, naturally high arsenic background levels)	Mean \pm SD	24400 \pm 17000	VÁZQUEZ et al., 2013
		Range	6000-60000	
	Control (Buenos Aires, approx. 30 km away)	Mean	3500	
		Range	3000-4000	
Australia (urban area)	Sydney (areas near transport corridors and with industrial past)	Mean \pm SD	70 \pm 40	JAFARI, 2016
		Range	30-200	
	Control (area north of Sydney)	Mean \pm SD	110 \pm 100	
		Range	30-32	
Romania	CAD	Mean \pm SD	1080 \pm 230	BADEA et al., 2016
	Control-healthy	Mean \pm SD	1040 \pm 230	
Romania	MN		< detection limit	BADEA et al., 2018
Control-healthy	Mean	840		
Finland	Diet exposure	Mean \pm SD	143 \pm 39	ROSENDAHL et al., 2020
		Range	100-210	
	Control	Mean \pm SD	86 \pm 24	
		Range	60-120	
Finland	Clinically healthy	Mean \pm SD	20 \pm 20	ROSENDAHL et al., 2022
		Range	10-90	
Finland	Epileptic dogs	Mean \pm SD	400 \pm 780	ROSENDAHL et al., 2023
	Control-healthy	Mean \pm SD	50 \pm 80	

¹CAD- canine atopic dermatitis; MN- mammary neoplasms; SD- Standard deviation

Healthy dogs from Finland (ROSENDAHL et al., 2022; ROSENDAHL et al., 2023) had similar arsenic hair levels to dogs from control group from this study. The mean hair arsenic level of the exposed group (this study) was 105 times lower than that of the dogs living in the Las Alamos area (Argentina) with a naturally high background levels of arsenic in soil and water (VAZQUEZ et al., 2013). The highest arsenic level in water from Las Alamos ($75 \mu\text{g As L}^{-1}$) by far exceeded the $10 \mu\text{g L}^{-1}$ WHO limit for arsenic in water for drinking, but no correlation was found between the arsenic levels in hair and in groundwater suggesting other environmental factors influencing the exposure (RODRIGUEZ CASTRO et al., 2013). According to KURTTIO et al. (1998), an increase of $10 \mu\text{g As L}^{-1}$ of drinking water or an increase of 10-20 μg of arsenic per day in food corresponded to a $100 \mu\text{g kg}^{-1}$ increase in human hair arsenic level. Healthy dogs from Romania (BADEA et al., 2016, BADEA et al., 2018) had a mean arsenic level 4-5 times higher than the one measured in dogs from the exposed group of this study. Dogs exposed to arsenic from rice in their diet ($\geq 80\%$) had 1.6 times lower hair levels than dogs from the exposed Salsigne group (this study), while levels were similar for dogs from control groups from both studies (ROSENDAHL et al., 2020).

As there is a paucity of arsenic level data in nails of dogs, in Table 8 we included data for different animal species such as snowshoe hare, muskrat, red squirrel and humans, residing in contaminated mining areas for comparison. In this study, three dogs had arsenic nail values above $1000 \mu\text{g kg}^{-1}$ (limit for normal values in human hair and nails; WHO, 1983; ATSDR, 2007). Two of them were accompanied by high hair arsenic content. Low number of samples probably disabled us to notice significant differences in nail arsenic levels between the exposed and control group. Mean nail arsenic level in dogs from the exposed group was two times lower than mean level found in nails of snowshoe hares near the Giant Gold Mine in Canada (AMUNO et al., 2018). Similarly, dogs from control group (this study) had two times lower mean arsenic than Canadian snowshoe hares from control area (AMUNO et al., 2018). Range of nail values in dogs (exposed group, this study) was comparable to the range of values found in red squirrels residing the Giant Gold Mine (JAMWAL et al., 2023). The nail arsenic levels in dogs from the exposed group (this study) were comparable to humans' residing in the tungsten Panasqueira Mine area, Portugal ($650 \mu\text{g kg}^{-1}$; COEHLO et al., 2014). The earlier study from the same area showed somewhat lower arsenic nail levels ($500 \mu\text{g kg}^{-1}$; COEHLO et al., 2012). Mean arsenic nail levels in humans living near former arsenic mine in Devon, UK (BUTTON et al., 2009) were seven times higher than in Salsigne dogs from our study, while levels in control groups of both studies resembled each other.

Table 8: Summary of literature data reported for arsenic level in nails of different animal species and humans ($\mu\text{g kg}^{-1}$)

Species	Location	Groups		As concentration	Reference	
Dog	France	Study (Salsigne old mining district)	Mean±SD	722±815	This study	
			Median	175		
			Range	84.4-1952		
		Control	Mean±SD	137±87.0		
			Median	125		
			Range	30.4-285		
Snowshoe hare	Canada	Exposed area (1-3 km near Giant mine, gold mine)	Mean±SD	1930±990	AMUNO et al., 2018	
			Range	1080-4000		
		Control (50-100 km away from mine)	Mean±SD	310±310		
			Range	47-936		
Muskrat	Canada	Exposed area (1-3 km near Giant gold mine)	Range	660-2100	JAMWAL et al., 2023	
			Control (50-100 km away from mine)	Range		<50-63
		Exposed area (1-3 km near Giant gold mine)	Range	ND-1400		JAMWAL et al., 2023
			Control (50-100 km away from mine)	Range		
Human	Australia	Exposed (historical gold mining area, children)	Mean	490	PEARCE et al., 2010	
			Range	150-2100		
Human	United Kingdom	Exposed (historical arsenic mining area, residents)	Mean	5406	BUTTON et al., 2009	
			Range	858-25981		
		Control (approx. 400km away from mine)	Mean	122		
			Range	73-273		
Human	Portugal	Environmentally exposed (active tin-tungsten mining area, residents in proximity)	Mean	500	COELHO et al., 2012	
			Occupationally exposed (mine workers)	Mean		750
		Control (less than 5km from mine but living in non-contaminated areas and not exposed professionally)	Mean	220		
			Mean	220		
Human	Portugal	Environmentally exposed (active tin-tungsten mining area, residents in proximity)	Mean	650	COELHO et al., 2014	
			Occupationally exposed (mine workers)	Mean		1010
		Control (less than 5km from mine but living in non-contaminated areas and not exposed professionally)	Mean	1010		
			Mean	220		
Human	Australia	Historical gold mining area, residents			MARTIN et al., 2013	
			2006 study	Mean		464
			2011 study	Range		150-2100
				Mean		171
			2013 study	Range		30-540
				Mean		173
Human	World	Exposed and control	Range	0-68300	SIGNES-PASTOR et al., 2021	
			Range	0-68300		

Nails in residents of former gold mining regions in Australia exceeded the ones found in Salsigne dogs (this study) by 1.5 (PEARCE et al., 2010) and four times (MARTIN et al., 2013), respectively.

Our findings of similar arsenic levels in hair and nails between the sexes corroborated the results reported in dogs by ROSENDAHL et al. (2020). On the other hand, VÁZQUEZ et al. (2013) observed 65% higher arsenic hair levels in female dogs exposed to naturally high background arsenic in the environment compared to males. The cause was attributed to differences in hair keratin structures between the sexes, as well as variations in metabolism between males and females (VAHTER et al., 2007).

Absence of associations between arsenic in hair and nail, and age, was in line with observations of ROSENDAHL et al., 2020. In contrast, JAFARI (2014) found that arsenic level in hair of dogs from urban areas in Australia had a strong positive association with age. Human studies also suggested that arsenic tends to accumulate with age (RAVAULT et al., 2002; ATSDR, 2007; INERIS, 2010). The length of time that dogs resided in the Salsigne area also showed no influence on arsenic levels in hair or nail. Out of 27 dogs, 7 (26%) were born in the Orbiel Valley and have lived in the same or close areas since. Further, 12 (44%) dogs moved to the valley as puppies and have lived the rest of their life in the same area. Swimming in local rivers of the Salsigne district had no influence on levels of arsenic in hair and nails of dogs. Although water and sediments in local rivers contain high levels of arsenic (KHASKA et al., 2018; GIRARDEAU, 2019; DELPLACE et al., 2022), arsenic does not readily penetrate the cutaneous barrier so the absorption through skin was considered a negligible route of absorption in humans (ATSDR, 2007).

We did not find any association between body mass and arsenic levels in hair or nails of dogs in this study. Scarce data on this topic suggested that arsenic could play a role in increased Body Mass Index (BMI) in humans and laboratory animals (EICK and STEINMAUS, 2020).

The concentration of arsenic in hair and nails of dogs from this study was found to be highly positively correlated. Evidence from human studies indicates that arsenic in toenails correlates with hair and fingernail concentrations, primarily in highly exposed population with mean/median toenail arsenic $\geq 1000 \mu\text{g kg}^{-1}$ (SIGNES-PASTOR et al., 2021). We noticed that arsenic levels in hair of dogs living in the same households (e.g., E1, E2, E3, and E4; E10 and E11; E13 and E14; E16, E17, and E18; C10 and C11; C19 and C20) were similar, in both

exposed and control group. Among dogs from the exposed group, several dogs came from the same neighborhood in Conques-sur-Orbiel and showed the highest levels of arsenic in both hair and nails. This could be explained by the fact that this specific neighborhood was impacted by the 2018 floods with the deposits of potentially contaminated sediments in the direct living environment of the dogs.

Although there is a correlation between arsenic exposure and a number of human cancers, such as respiratory cancers and skin carcinomas (EISLER, 1988; ATSDR, 2007), studies attempting to document arsenic-related cancers in dogs have yielded inconclusive results (GARLAND, 2018). Nevertheless, several adverse effects of arsenic in humans have been corroborated in animals, including cardiotoxicity, neurotoxicity and immunotoxicity (SU et al., 2023). The available evidence indicates that exposure to arsenic, particularly iAs, can induce a variety of adverse effects in dogs, including ulcerative dermatitis, liver damage, chronic renal disease, and the development of myocarditis (KIM et al., 2018). Four dogs in the exposed group suffered from chronic conditions, all dermatological in nature, as well as neurological for one dog among those. The first one already developed clinical signs before moving to the exposed area. The second had rashes during stressful situations, while the third suffered from atopic dermatitis, food allergies and intestinal issues accompanied with abdominal pain. For the fourth dog, the owner reported only epilepsy-like clinical signs without an official diagnosis by a veterinarian, but dermatitis was observed during sampling by the author. This dog later died at the beginning of 2024 from unknown causes. Although arsenic has been linked to dermatological conditions in both humans and dogs, we cannot state with certainty that environmental exposure to arsenic had impact on the health status of these dogs, while hair levels of these dogs were below the mean of the exposed group. In the literature, arsenic has been found to induce adverse health effects of pet dogs by negatively affecting the immune, cardiovascular and neurological systems as well as leading to, among others, myocarditis, chronic renal disease and liver damage (KIM et al., 2018; SU et al., 2023). As previously stated by MCCAULEY et al. (2020), long-term arsenic exposure may result in the depletion of taurine, potentially leading to the emergence of dilated cardiomyopathy. Most of these effects may appear, as in humans, after an extended period and may not be recognized as a linked consequence of arsenic exposure. However, there is still a paucity of data on possible health effects linked to arsenic exposure in dogs and more data is needed (SQUADRONE et al., 2017). It is important to note that levels in the hair and nails of dogs included in this study represented levels from the period of at least 30 days before sampling.

Finally, variables that may have influenced this study's results include different sampling month, during which exposure may have varied, the ratio between newly grown and old hair in dogs sampled during shedding periods, variation of habits during different seasons, as well as the frequency of grooming in dogs.

6. CONCLUSIONS

- Dogs from exposed group had higher arsenic levels in hair than dogs from control group.
- Hair of dogs was proved as reliable marker of environmental exposure to arsenic in a former mining area.
- Age, sex, body mass, residence period in the Salsigne area and swimming habits in the exposed group did not influence the arsenic levels in hair and nails.
- High positive correlation was found between arsenic levels in hair and nails.
- Arsenic levels in the hair of the dogs in the present study were generally aligned with the literature data for dogs but was much lower than the data reported for dogs from Argentina, Romania and laboratory diet-exposed dogs.
- Arsenic levels in the nails of the dogs in the dogs in the present study from the Salsigne area were similar to those detected in wild mammals and humans living near former mining areas.

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8. SAŽETAK

Razina arsena u dlaci i noktima pasa s područja nekadašnjeg rudnika te kontrolnog područja u Francuskoj

Abigail Rose Lily Plançon

Nekada najveći u Europi, rudnik zlata u Salsigne (jug Francuske) prestao je s radom prije dvadeset godina. Značajne količine otpadnog materijala koje su zaostale nakon zatvaranja rudnika izložene su vjetru, kiši i ispiranju, što rezultira suhim i mokrim taloženjem sitnih čestica metal(oid)a (arsena, olova, bizmuta, bakra, kadmija, žive i cinka) u okoliš. Studije utjecaja izloženosti domaćih životinja, kućnih ljubimaca i ljudi s tog područja spomenutim onečišćivačima vrlo su rijetke bez obzira na potencijalni rizik za zdravlje.

Istraživanja i važnost povezanosti zdravlja ljudi sa zdravljem životinja i okoliša dio su One Health koncepta. Povezane studije pokazale su da psi kao kućni ljubimci mogu poslužiti kao pouzdani indikatori dugotrajne izloženosti zagađenju iz okoliša. Stoga su u ovoj studiji prikupljeni uzorci dlake (N=49) i noktata (N=14) pasa s područja nekadašnjeg rudnika zlata i arsena (u radijusu od oko 10 km od rudnika Salsigne) te kontrolnog područja u centralnoj Francuskoj (u radijusu od oko 12 km sjeverozapadno od Lyona). Vlasnici pasa dali su pisani pristanak za sudjelovanje pasa u studiji te biometrijske podatke o psima, njihovim prehranbenim navikama i ponašanju izvan kuće. U dlaci pasa koji žive u blizini rudnika nađene su više razine arsena u odnosu na dlaku pasa s kontrolnih područja, dok nokti nisu pokazali takvu razliku. Spol, dob, tjelesna masa, godine koje je pas proveo na širem području rudnika te navika plivanja u rijeci Orbiel i njenim pritokama nisu pokazale utjecaj na razinu arsena u dlaci i noktima. Nađena je jaka povezanost između razina arsena u dlaci i noktima pasa. Rezultati ovog istraživanja pokazali su da razina arsena u keratiniziranim tkivima pasa koji dijele kućanstvo s ljudima može poslužiti kao vjerodostojan pokazatelj onečišćenja arsenom na području opterećenom posljedicama višestoljetnih rudarskih aktivnosti.

Ključne riječi: onečišćenje arsenom, psi, dlaka, nokti, bivše rudarsko područje

9. ABSTRACT

Arsenic content in the hair and nail samples of dogs living in an old mining district and control areas in France

Abigail Rose Lily Plançon

The Salsigne gold mine (South France), once the largest in Europe, ceased its operations twenty years ago. However, a considerable quantity of waste material exposed to wind, rain drainage, and flooding, leads to the dry and wet deposition of fine metal(oid) particles (arsenic, lead, bismuth, copper, cadmium, mercury and zinc) in the surrounding area. Nevertheless, there is a lack of exposure studies on domestic animals, pets, and humans in the region. The One Health concept posits that human health is deeply interconnected with the health of animals and their environment.

Pet dogs can serve as reliable sentinels of long-term exposure to environmental pollutants. Thereby, hair (N=49) and nails (N=14) were sampled from dogs residing in mining (up to 10 km from Salsigne mine) and control areas (central France, 12 km SW from Lyon). The owners of the dogs provided consent for their participation in the study, along with their animals' biometric data, dietary habits, and outdoor routines. Dogs residing in communes near the mine exhibited higher arsenic levels in hair compared to those from the control area, while in nails this difference was not confirmed. Sex, age, body mass, duration of residence in the mining region or regular swimming in nearby tributaries of the Orbier River had no influence on hair or nail arsenic levels. However, a strong correlation was observed between arsenic levels in hair and nails. This research has shown that arsenic found in the keratinized tissues of pet dogs living alongside humans can serve as a reliable indicator of arsenic pollution resulting from centuries of mining activity.

Key words: arsenic pollution, dogs, hair, nails, former mining area

10. CURRICULUM VITAE

I was born in Ecully, France on 19/11/1998. I graduated in 2016 from La Favorite Sainte-Thérèse highschool in 2016 with highest honour in my scientific *Baccalauréat*. After I graduated, I did one year of *classe préparatoire* BCPST and one year of doctor studies. In 2018, I enrolled at the Faculty of Veterinary Medicine of the University of Zagreb, Croatia, where I completed my studies in English and got the best student award in my generation for 6 years straight.

Throughout my studies, I completed several externships in France, mostly in small animal practices, farms and at Paris' veterinary school, Maisons-Alfort. This allowed me to broaden my mind and learn different techniques, develop my team spirit and further my knowledge. I also participated in my faculty's summer school about zoonoses in Dubrovnik. During one of my externships, I discovered an interest in Emergency and Critical Care Medicine, that I am currently pursuing by getting Recover certified.

Since I was young, I have done volunteer work in several fields, among others: wildlife rehabilitation in France and in Croatia, gardening at the faculty's community garden, helping at a shelter in Croatia.

Finally, my hobbies include reading, hiking with my dogs and of course, *kitsuke*.