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Option

Biotechnology and Molecular Pathology

Theme

Scincus scincus as a biomarker of environmental heavy metals and metalloids pollution and probable health impact of its consumption

By

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ABREVIATIONS

Abbreviations list

POPs	Persistent Organic Pollutants
DDT	Dichloro-diphenyl-trichloroethane
PCBs	Polychlorinated biphenyls
VOCs	Volatile Organic Compounds
WHO	World Health Organisation
DNA	Deoxyribonucleic acid
ROS	Reactive Oxygen Species
hCtr1	high-affinity human copper transporter
ATP	Adenosine Tri-Phosphate
MMA	Methylarsonic Acid
DMA	Dimethylarsinic Acid
OMS	Organisation Mondiale de la Santé
SC	Stratum Corneum
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAD^+	Nicotinamide Adenine Dinucleotide
MAPK	Mitogen-Activated Protein Kinase
SAPK	Stress-Activated Protein Kinase
SOD	Superoxide Dismutases
GSH	Glutathione
ATP	Adenosine Triphosphate
CDK	Cyclin-Dependent Kinases
NF-κB	Nuclear Factor-Kappa B
P53	Tumor Protein 53
MEG3	Maternally Expressed Gene 3
DAT1	Dopamine Transporter Gene
CRAPC	Center of Scientific research and Techniques in physicochemical Analyses
AAS	Atomic Absorption Spectrometry
SH	Src Homology
ZIP	Zinc Transporter Protein
ZnT1	Zinc Transporter 1
DMT1	Divalent Metal Transporter 1
SACs	Sacsin Molecular Chaperone
γ-GT	Gamma-Glutamyltranspeptidase

Summary

(*Scincus scincus*) consumption is a very common culinary practice in El Oued area, and is believed to deliver a good nutritional value, and therapeutic benefits. However, due to the lizard's physiological properties and ability to bioaccumulate heavy metals, it may represent a risk for its consumers instead of the claimed benefits. In order to investigate the capacity of *S.scincus* to accumulate heavy metals, the latter's tissues were analysed by atomic absorption spectrometry after a reflux acid digestion, along a histological study on kidneys, liver, muscles, and brain tissue. Results have shown the presence of the following metals: Cu, Zn, Pb, Mn, As, Cd, Cr, Co, Hg, Al, Se, and Ni. Most of them were present in significant amounts and exceeded WHO norms, with the exception of Cr. Liver and kidneys contained the highest quantities of Cu, Mn, Cr, Ni, Hg, Se, and Al, while muscle and skin tissues contained higher amounts of As, Cd, Zn, and Pb. The histological study results, revealed various tissular damage manifestations, namely: a remarkable kidney tissular and cellular damage, liver metabolic stress, brain tissue aberrancies, and muscle rifts. After comparative statistical analyses of daily intake of one sandfish, and/or five ones, and WHO norms, it has become clear that eating this skink is not safe, because it bioaccumulates heavy metals and humans consuming it may be exposing themselves to a chronic toxicity of these contaminants.

Keywords: Lizard, heavy metals, pollution, bioaccumulation, toxicity, health

Résumé

La consommation de *(Scincus scincus)* est une pratique culinaire très courante dans la région d'El Oued, ce dernier est cru d'avoir une valeur nutritionnelle importante, ainsi que des avantages thérapeutiques. Cependant, en raison des propriétés physiologiques du poisson sable et de sa capacité à bioaccumuler des métaux lourds, il peut représenter un risque pour ses consommateurs au lieu des bénéfices attendus. Afin d'étudier la capacité de *S.scincus* à accumuler les métaux lourds, les tissus de ce dernier ont été analysés par spectrométrie d'absorption atomique après une digestion acide à reflux, ainsi qu'une étude histologique sur les reins, le foie, les muscles et le tissu cérébral. Les résultats ont montré la présence des métaux suivants : Cu, Zn, Pb, Mn, As, Cd, Cr, Co, Hg, Al, Se et Ni. La plupart d'entre eux étaient présents en quantités importantes et dépassaient les normes de l'OMS, à l'exception du Cr. Le foie et les reins contenaient les plus grandes quantités de Cu, Mn, Cr, Ni, Hg, Se et Al, tandis que les tissus musculaires et cutanés contenaient des quantités plus élevées d'As, Cd, Zn et Pb. Les résultats de l'étude histologique ont révélé diverses manifestations de dommages tissulaires, à savoir : un dommage tissulaire et cellulaire remarquable des reins, un stress métabolique du foie, des aberrations du tissu cérébral et des failles musculaires. Après des analyses statistiques comparatives de la consommation journalière d'un poisson sable, et/ou de cinq, ainsi que des normes de l'OMS, il est devenu clair que la consommation de ce scinque n'est pas sans danger, car il bioaccumule les métaux lourds et les humains qui le consomment peuvent s'exposer à une toxicité chronique de ces contaminants.

Mots-clés : Lézard, métaux lourds, pollution, bioaccumulation, toxicité, santé.

ملخص

يعد استهلاك (_Scincus scincus_) ظاهرة شائعة جدًا في منطقة الوادي سوف ، ويُعتقد أنه يقدم قيمة غذائية جيدة وفوائد علاجية. ومع ذلك ، نظرًا للخصائص الفسيولوجية لسمكة الرمال وقدرتها على تركيز المعادن الثقيلة في جسمها، فقد تمثل خطرًا على المستهلكين بدلاً من الفوائد المزعومة. من أجل التحقيق في قدرة _S. scincus_ على تركيز المعادن الثقيلة ، تم تحليل أنسجة الأخير عن طريق جهاز الطيف للامتصاص الذري بعد هضم الانسجة بالاحماض ، إلى جانب دراسة نسيجية على الكلى والكبد والعضلات وأنسجة المخ. أظهرت النتائج وجود المعادن التالية: النحاس ، الزنك ، الرصاص ، المنغنيز ، ارسنيك ، الكادميوم ، الكروم ، الكوبالت ، الزئبق ، الألمنيوم ، السلينيوم ، النيكل. كان معظمهم موجودين بكميات كبيرة وتجاوزوا معايير منظمة الصحة العالمية ، باستثناء الكروم. احتوى الكبد والكلى على أعلى كميات من النحاس ، المنغنيز ، الكروم ، النيكل ، الزئبق ، السيلينيوم ، و الألمنيوم، بينما احتوت أنسجة العضلات والجلد على كميات أعلى من الارسنيك، الكادميوم ، الزنك، و الرصاص. أظهرت نتائج الدراسة النسيجية العديد من مظاهر تلف الأنسجة وهي: تلف خلوي و في الكلى ، إجهاد استقلابي للكبد ، انحرافات في أنسجة المخ ، وشقوق عضلية. بعد التحليلات الإحصائية المقارنة للاستهلاك اليومي لسمكة رمل واحدة و / أو خمسة سمكات ، ومعايير منظمة الصحة العالمية ، أصبح من الواضح أن تناول هذا النوع من السحليات ليس آمنًا ، لحدوث تراكم بيولوجي للمعادن الثقيلة ، وقد يؤدي تناول البشر له إلى تعريض أنفسهم لخطر صحي من هذه الملوثات.

الكلمات المفتاحية: سحلية ، معادن ثقيلة ، تلوث ، تراكم بيولوجي ، تسمم ، صحة

INTRODUCTION

Introduction

Culinary practices have long been associated to people's culture and are mostly inherited throughout generations. They are usually trusted to not cause any harm, because they were passed down by the ancestry. An interesting Algerian culinary tradition that is mostly practiced by people in El Oued region, is the consumption of a desertic specie referred to as "Cherchemen" locally, the "sandfish" commonly, and (*Scincus scincus)* scientifically. According to a survey on 633 consumers, about 50% revealed consuming it as a low-price protein source, while around 20% mentioned eating it for its believed medicinal properties. Due to its widespread consumption, this specie gained a socioeconomical and cultural importance within the Soufi population (Toumi et al., 2017). *S. scincus* has long been perceived to have therapeutic virtues, mainly aphrodisiac properties (Toumi, 2018). However, there are no reliable proofs that demonstrate how consuming this animal may help fertility, or provide any therapeutic benefit.

Parallelly, squamates (including sandfish) have been known to possess a high potential as bioindicators of environmental pollution. Because, these species are ectotherms and have a low-rate metabolism, making them more vulnerable to environmental pollution, in comparison to mammals and birds. Additionally, they have a simple enzymatic system, and a weak ability to detoxify their body from pollutants, which leads to a fast manifestation of adverse effects that are linked to pollutants (Campbell and Campbell, 2000).

Bioaccumulation takes place when an organism's balance of a toxic substance absorption and elimination is challenged, making the organism unable to excrete the absorbed material. As a result, the chemical substance concentration inside the organism will exceed the one found in the organism's environment (Hill, 2010). Due to lizards being on the secondary predation position in the food chain, they may bioaccumulate pollutants that contaminate their preys. Since they are insectivorous, their preys are low on the food chain, and may bioaccumulate chemical substances of both metallic and organic nature (Silva et al., 2020). With the increase of the toxic substance in the food chain, the sandfish body may start to biomagnify the toxicants.

Previous studies on the consumption of *S. scincus* flesh have all neglected the potential risks of lizard consumption, and only focused on its nutritious value and claimed therapeutic

effects. Certainly that, the area of El Oued Souf is known to encounter recurrent challenges with environmental pollution. Which leads us to question whether it is safe or not to consume an organism that is known to accumulate environmental pollutants? Especially that, *S. scincus* is known to live up to 8 years (Ellis et al., 2011), thus, old ones may have bioaccumulated and magnified years' worth of contaminants.

Heavy metals are persistent inorganic contaminants that stay in soils for prolonged periods of time, these elements present high toxicity to humans, sometimes at very low levels. The primary exposure route to heavy metals is through food and water, which are under some conditions contaminated by these metals, such as arsenic, cadmium, nickel, mercury, and lead. Some studies have indicated that lizards accumulate heavy metals and could be used as bioindicators of heavy metal pollution (Campbell and Campbell, 2000). Making *S.scincus* one of the potential candidates that could expose humans consuming it to the different heavy metals they may accumulate. Chronic exposure to heavy metals, even at very low levels, could induce diverse health issues, such as renal injury, neuronal damage, increase the risk of cancer, and may induce cardiovascular disorders (Rehman et al., 2018).

Due to the available contradictory data, it is of a great necessity to investigate the safety of consumption of this lizard, and whether the widespread long claimed therapeutic benefits are realistic.

CHAPTER I

REVIEW OF LITERATURE

I. Environmental pollution

Environmental pollution could be defined as "the contamination of the physical and biological components of the earth/atmosphere system to such an extent that normal environmental processes are adversely affected" (Muralikrishna and Manickam, 2017).

A contaminant or a pollutant could be termed "xenobiotic", which is by definition "Any compound not occurring within the normal metabolic pathways of a biological system" (P. Talcott, 2012). Xenobiotics are chemical substances that are found but not produced in organisms or the environment. Some naturally occurring compounds could be considered xenobiotics when present in higher concentration than the established norms. Such substances include: heavy metals, pesticides, drugs, food additives, polychlorinated biphenyls, and certain natural compounds when presented in excess of natural levels.

Environmental pollution occurs when there is an environmental incapacity to neutralize harmful compounds resulting from human activities in due course, without any structural or functional damage to the system (Muralikrishna and Manickam, 2017).

I.1. Categories of pollutants

There are three main types of environmental pollution, namely: Air, water, and soil pollution. Any chemical or material from either human or natural sources can pollute the environment. Thus, pollution could be induced by many types of pollutants, as indicated in table 1. Pollutants of natural sources are often described as chemicals or materials containing carbon atoms. They are referred to as "Natural chemicals" because they are produced naturally by animals, microorganisms, and plants. On the other hand, Inorganic chemicals — That are not considered natural pollutants—differ from organic chemicals by not containing carbon atoms (Spellman, 2010).

I.1.1. Organic pollutants

They are referred to as "Persistent organic pollutants (POPs)", and are described by the European Commission of Environment as "chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment. This group of priority pollutants consists of pesticides (such as DDT), industrial chemicals (such as polychlorinated biphenyls, PCBs) and unintentional by-products of industrial processes (such as dioxins and furans)."

I.1.2. Inorganic chemicals

They consist of heavy metals (such as lead), metalloids (such as arsenic), Acids (such as sulphur dioxide), base (such as ammonia), and others.(Borah et al., 2020).

I.1.3. Physical pollutants

Consist primarily of solid materials found in inappropriate locations (such as trash). High temperature water released from industrial plants could also be considered a physical pollutant (Spellman, 2010)

I.1.4. Radioactive chemicals

Could be naturally found in rocks, water, and soil. These pollutants could also emerge from human-anthropogenic activities, such as radioactive waste storage sites. (Spellman, 2010)

I.1.5. Biological pollutants

Are represented by pathogenic microorganisms, such as fungi, worms, bacteria, protozoa and viruses (Spellman, 2010). It is important to note that even dead microorganisms and parts of organisms can pollute air, water and, soil (Spellman, 2010).

I.1.6. Acid pollutants

Could either be organic, or inorganic. An example of acid pollution is that one induced by nitric and sulfuric acid, which are commonly found in acid rain (caused by air pollution).

I.1.7. Organo-metalloid pollutants

They have metal/metalloid-carbon bond, that are usually covalent and reached between soft acid metals and soft ligands. These compounds are found to be persistent, easily concentrated, and highly toxic (Haydee and Dalma, 2017).

Table 1. Pollutants types (Hill, 2010)

Category	Examples
Organic chemicals	Polychlorinated biphenyls (PCBs), oil, many
Inorganic chemicals	pesticides
Organometallic chemicals	Salts, nitrate, metals and their salts
Acids [a]	Methylmercury, tributyltin, tetraethyl lead

Physical [a]	Sulfuric, nitric, hydrochloric, acetic
Radiological [a]	Eroded soil, trash
	Radon, radium, uranium
Biological [a]	Microorganisms, pollen

[a] Acids, as well as physical and radioactive pollutants can be either organic or inorganic. Sulfuric acid is inorganic, while acetic acid (found in vinegar) is organic. Biological pollutants are mostly organic, but contain inorganic components.

I.2. Sources, transport, and transformation of pollutants

I.2.1. Sources

In general, pollutants emerge from anthropogenic activities, motor vehicles (cars, buses, airplanes, ships, and off-road vehicles), chemical and petroleum refineries, manufacturing facilities, commercial operations (such as: dry cleaners, and bakeries), plants generating electric power by burning coal, oil or natural gas, Agricultural operations growing crops or raising animals, food processing, municipal operations (such as drinking water/waste water treatments), road maintenance, activities occurring in commercial/municipal buildings, and private dwellings (such as consumer product usage) (Hill, 2010).

Pollutants could emerge either from direct or indirect sources: (a) Direct sources are represented by pollutants that are directly disposed of in the surrounding environment. Such as: phenols from pharmaceutical industries, hydrocarbons of petroleum effluent, slowly degrading plastics, persistent dyes, pesticides and insecticides (organophosphorus compounds, benzimidazoles, methyl parathion, and morpholine), and paper and pulp effluent, which degrade slowly with a direct effect on the environment.(Mathew et al., 2017). (b) Indirect sources represent pollutants that are released into the environment through a secondary source, these include hospital discharge such as pharmaceutical products, anti-inflammatory drugs (non-steroidal drugs), pesticides, and herbicides that are released into the soil. These chemicals are considered indirect pollutants both in their original and fragmented state (Mathew et al., 2017).

I.2.1.1. Water pollution sources

Water contaminants could emerge from: domestic wastes, insecticides and herbicides use, food processing waste, volatile organic compounds (VOCs) such as formaldehyde, heavy metals, chemical waste, persistent organic pollutants, oil, nutrient pollutants (nitrogen, phosphates) (Muralikrishna and Manickam, 2017), and so on.

I.2.1.2. Air Pollution sources

Air pollutants mainly emerge from: sulfur dioxide, nitrogen dioxide, carbon monoxide, ozone, volatile organic compounds (such as toluene, xylene, and benzene), airborne particles with radioactive pollutants, fossil fuel combustion (Muralikrishna and Manickam, 2017), and so on.

I.2.1.3. Soil Pollution sources

Soil pollutants mainly include contaminants from industrial and domestic activities. These commonly include: hydrocarbons (such as petroleum and paraffin), solvents (such as methanol and acetone), and inorganic compounds (such as heavy metals) (Muralikrishna and Manickam, 2017).

Fossil fuel pollutants commonly emerge from power-generating plants, petroleum refineries, petrochemical plants, fossil fuels production and distribution, road transport, shipping and aircraft industries (Muralikrishna and Manickam, 2017).

I.2.2. Transport and transformation of pollutants

It is important to know that pollutants contaminating soil, water, and air are interrelated. They rarely stay at their source, and are transported through air, water, soil, and sediment, and often food. Xenobiotics often move across national and international boundaries (transboundary), traveling along with air, water, and carried in animal tissues (biotransport), such as salmon, whales and migratory birds (Hill, 2010).

Pollutants are usually transformed into an end product, different form the initial one they were emitted in. The resulting chemical product may be no longer considered a pollutant. An example of this could be the degradation of a biological pollutant by microorganisms and its incorporation within. In contrast, a molecule like "dioxin" can take several years to be transformed into a non-toxic form (Hill, 2010). Organic contaminants are oxidized to carbon (IV) oxide by microorganisms, however, most metals do not undergo degradation by microorganisms (Speight, 2017). Which makes heavy metal contamination of soil presents many risks to human health and the ecosystem. These risks can reach humans either through the food chain (soil-plant-human or soil-plant-animal-human) or through direct exposure by dermal contact or ingestion of soil contaminants (soil-human or soil-water-human). Environmental risks are presented through phytotoxicity in eco-toxicity to soil fauna and flora (McLaughlin et al., 2000).

I.3. Heavy metals and metalloids as inorganic pollutants

"Among the 35 natural existing metals, 23 possess high specific density above 5g/cm^3 with atomic weight greater than 40.04 and are generally termed heavy metals." (Azeh Engwa et al., 2019). They have a high atomic weight or density, those that are most found at contaminated sites are: lead (Pb), chromium (Cr), arsenic (As), Zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni). Metalloids, on the other hand, own intermediate properties, between those of metals and nonmetals (fig.1).

Figure 1. The periodic table

While organic pollutants can be degraded, heavy metals cannot. Consequently, they remain present in the environment for years after the removal of their point sources (Gall et al., 2015). Heavy metals and metalloids contamination of soils occurs through emissions from increased industrial development and fast industrialization. In addition to mine tailings, disposal of high metal wastes, leaded gasoline and paints, increased usage of fertilizers and pesticides, animal manures, sewage sludge, wastewater irrigation, residues of coal combustion, spillage of petrochemicals, and atmospheric deposition of airborne pollutants (Speight, 2017). Table 2 suggests contamination risk pathways that could explain how contaminants can reach humans.

Table 2. Suggested contamination risk pathways of metals and metalloids (McLaughlin et al., 2000)

Element	Dominant risk pathway	Secondary risk pathway
As	Soil ingestion by animals/humans	Food chain transfer
Cd	Food chain transfer	Phyto- and ecotoxicity
Cr	Phyto- and ecotoxicity	Metal leaching
Cu	Phyto- and ecotoxicity	Soil ingestion by animals/humans
Ni	Phyto- and ecotoxicity	Soil ingestion by animals/humans
Pb	Soil ingestion by animals/humans	Phyto- and ecotoxicity
Zn	Phyto- and ecotoxicity	Food chain transfer

II. Potential health complications induced by pollutants

Toxicity can be defined as "An adverse health effect caused by a drug. The adverse effect can range from a minor unpleasant side effect to a major threat to the life quality, health, or survival of the patient" (H. Kerns, 2016). A toxicant is the substance inducing the adverse effect in a plant, animal, or human. That is by impairing vital metabolic processes (Hill, 2010). Pollutants' toxicity on the environment has introduced hazardous effects to humans, plants, and the ecosystem—Particularly trace elements such as heavy metals. Certain chemicals are distinctly persistent, accumulate in human tissues, and have the capacity of causing human health affections (Tchounwou et al., 2012). It is also important to note that a pollutant's effect relies on a lot of parameters, including: the dose, time and duration of exposure, age, gender, and health of the exposed animal or human (Hill, 2010).

II.1. Pollutants' time and exposure effects

Induced toxicity by pollutants could be acute, sub-chronic, or chronic.

II.1.1. Acute toxicity

Is an adverse effect that arises shortly after exposure to a chemical and if death did not occur, a complete recovery is achieved (L.Gadaga, 2014).

II.1.2. Sub-chronic toxicity

occurs when a toxicant causes adverse effects for a period exceeding one year, but less than the lifetime of the organism (M.Stöppler, 2021).

II.1.3. Chronic toxicity

It takes place after long-term or repeated exposure to a chemical at low doses. It is an adverse effect persisting long after the exposure to the pollutant has ended. Long term could be several weeks, up to 40 years (Hill, 2010).

II.2. Pollutants' dose effects

"Anything is toxic at a high enough dose" (Hill, 2010). The dose-response relationship can be defined as "the measurement of the relationship between the dose of a substance administered and its overall effect (the response), either therapeutic or toxic. The dose–response relationship is based on observed data from experimental animal, human clinical, or cell studies. Generally, the higher the dose, the greater the response."(P.Ambery, 2013).

Figure 2 illustrates a dose-response curve, as the dose of the chemical increases, the adverse effect linked to it increases too. It is important to note that the time period of exposure is as important as the dose (Hill, 2010), when it comes to the adversity of the effect.
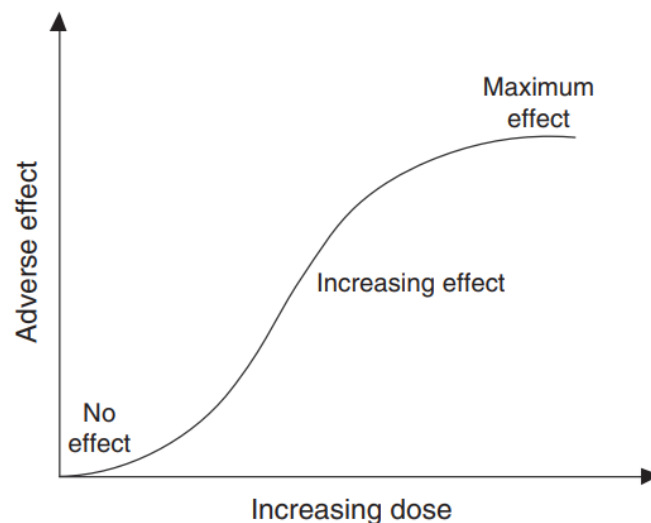
Figure 2. Increasing adverse effect with increasing dose (Hill, 2010).

II.3. Multiple exposure effects

Humans, animals and plants are exposed to more than one chemical in their environment (Hill, 2010). These multiple exposures could result in:

- **Additive effect:** is the combined effect of two or more chemicals equating the sum of the effect of each agents given alone, without them interacting in a direct way (CCOHS, 2019).

▪ **Synergism:** in toxicology, synergism is described as "the effect caused when exposure to two or more chemicals at one time results in health effects that are greater than the sum of the effects of the individual chemicals." (CCOHS, 2019)

▪ **Antagonism:** occurs when one agent interferes with the action of another one (Hill, 2010). Thus, the combined effect of the two agents is less toxic than the individual effect of each one (CCOHS, 2019).

II.4. Toxicity pathways

A toxicant exerts its effect on a systemic, or local level. Initially, the chemical is absorbed into the body through: lungs, gastro-intestinal tract, or skin. After internalization, the toxicant "may be absorbed (A) into the bloodstream, distributed (D) throughout the body, metabolized (M) by the body's tissues into different chemicals, and finally excreted (E) from the body". This trajectory could be abbreviated by[2] the acronym ADME (Hill, 2010) (fig. 3).
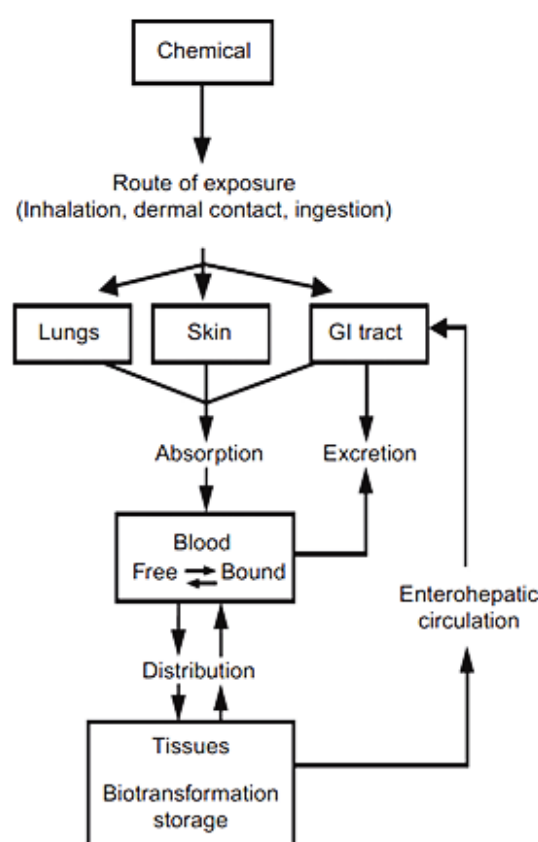
Figure 3. Schematic representation of potential toxicokinetic pathways after chemical exposure (Gupta, 2016)

II.4.1. Absorption

For a systemic response to occur, the toxicant has to be absorbed into the body. This process could happen by inhaling the toxicant into the lungs, ingesting it into the digestive system, or absorbing it through the skin (Hill, 2010). A chemical can also be introduced to the body through unusual ways, such as injection (subcutaneous, intravenous, and intramuscular). The administration process happens through the following routes:

- **Inhalation:** Where the toxicant is inhaled into the respiratory system, passing through the lung's alveoli into the bloodstream. Making inhalation the second fastest route (after injection) that delivers the chemical into the blood (Hill, 2010).

- **Ingestion:** is achieved through the oral administration (drinking/eating) of the toxicant into the digestive tract. Subsequently, the xenobiotic is digested into molecules, which may be absorbed through the wall of the small or large intestines into the bloodstream. Usually, absorption takes place in the small intestine. The chemical is absorbed to the portal blood system, through which the molecules reach the liver. The latter, is the first organ receiving the toxicant; without it being diluted in the blood; thus, the liver receives the highest dose of the toxicant (Hill, 2010). Conversely, the other organs receive a dose that is diluted in the blood.

- **Skin absorption:** dermal absorption consists of the transport of a chemical from the skin's outer surface into the internal layers of it, and into the body. Dermal absorption rate depends on the stratum corneum (SC) or the epidermis, which functions as a barrier protecting the deeper layers of the skin. Factors that influence the dermal absorption are (NIOSH, 2020):
  - Skin integrity;
  - Exposure site (temperature of the skin, thickness and hydration of the epidermis. It is important to note that the thinner the skin, the more permeable it is (Hill, 2010)
  - Physico-chemical properties of the toxicant;
  - Exposure duration (The longer the skin is exposed the greater is the absorption)
  - The surface area affected by the chemical (the larger the area, the more chemical amount is absorbed)

Toxicant uptake by the layers of the skin is done by diffusion, which is modulated by Fick's law. The latter enunciates that the diffusion rate across a barrier will be directly proportional to the concentration gradient (Semple, 2004). Thus, the chemical in contact with

the stratum corneum generates a gradient of concentration between the epidermis and the dermis producing a mass transfer (Semple, 2004).

There are three proposed mechanisms for chemical diffusion into the skin (NIOSH, 2020) (Fig. 4-6):
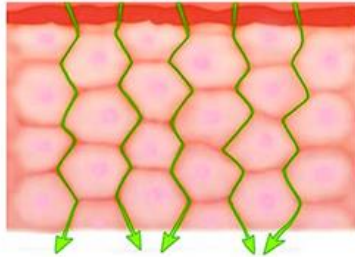
Figure 4. Intercellular lipid pathway
Some chemicals diffuse through the lipid-filled intercellular spaces between corneocytes (cells of the stratum corneum).
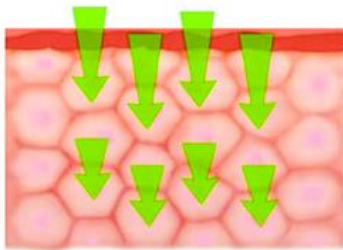
Figure 5. Transcellular permeation
The chemical diffuses through corneocytes, and cell-to-cell permeability.
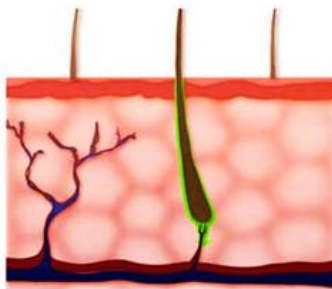
Figure 6. Appendages' pathway
The chemical may diffuse into the skin through skin appendages, namely: hair follicles, and glands. This pathway may be taken by the chemical during the initial stage of absorption.

II.4.2. Distribution

After absorption, the chemical agent is distributed within the body by the blood. The agent is then taken by organs to varying degrees. A particular chemical may exhibit the most prominent effects on its target organ(s). For a chemical to be toxic, it must attain its most vulnerable tissue at a high enough dose to induce an adverse effect (Hill, 2010).

II.4.3. Metabolism

Metabolism encompasses all chemical reactions occurring within the body to maintain life (Blanco and Blanco, 2017). There are two types of metabolic processes:

- Anabolism: Consists of the synthesis of new molecules using simpler ones. It is an endergonic process (requires energy to start, and stores it). It involves reduction processes that uses NADPH as a hydrogen ion provider (Blanco and Blanco, 2017).

- Catabolism: Consists of breaking down large molecules into smaller ones. It is an exergonic process (doesn't require energy to start, but releases it) and consists of oxidations using coenzymes, such as NAD+ as an electron receiver (Blanco and Blanco, 2017).

Xenobiotics are usually bio-transformed and metabolized into excretable metabolites, in order to expel them from the body. The major detoxification organs are the liver and kidneys, which are particularly active in this function. The chemical agent is mostly metabolized into a less toxic metabolite, however, in certain cases it could be metabolized into a more toxic agent. Such as the conversion of one benzene into benzene oxide, which damages the bone marrow (Hill, 2010).

II.4.4. Excretion

Excretion can be described as "A process by which toxicants and/or their metabolites are irreversibly transferred from the body to the external environment." (Gupta, 2016). Thus, by the end of exposure or its decrease, the concentration of the absorbed pollutants starts to lower (Hill, 2010). The extent of excretion depends on many parameters, such as how was the toxicant stored in the body.

Excretion comprises all the processes that lower the quantity of parent chemical compounds in the body, including biotransformation procedures. It is important to note that excretion processes that are carried by organs other than the kidneys are termed "extrarenal" or "nonrenal" excretion (Gupta, 2016).

- **Renal excretion**

The most important excretion route of toxicants and their metabolites is the renal excretion route. Renal excretion of xenobiotics occurs through three distinct mechanisms:

- Glomerular filtration
- Active tubular secretion ionized substances
- Passive tubular reabsorption of unionized substances

The glomeruli filter's the blood's plasma containing xenobiotics and produces an ultra-filtrate of the plasma containing different metabolites. Once the chemical is filtered through the glomeruli, it passes to the tubular lumen so that it is excreted, however, it may be

reabsorbed in a passive way across the tubular cells of the nephron into the bloodstream, thus, the compound would not be excreted in urines. Lipid-soluble ionized molecules, such as conjugated xenobiotics are poorly reabsorbed, hence, more easily excreted than their precursors through kidneys. Chemicals can be excreted passive diffusion from the plasma to urine through the tubule. The distal tubule acts as a lipoprotein barrier, favouring the transfer of lipid-soluble, and nonionized molecules. Thus, lipid soluble agents that are not ionized in the glomerular filtrate are reabsorbed into the bloodstream, contrastingly, those elements with low lipid solubility are partially reabsorbed. The rate of renal excretion of weak acids and bases is influenced by the pH of urine, as well as the affinities of electrolytes. The excretion degree of one chemical could be reduced by the administration of another, due to their competitive affinities. A new born and premature infants have an incompletely developed kidneys, thus, chemicals are not excreted fast enough, making them more toxic to new born than adults (Gupta, 2016).

■ **Biliary excretion**

The gastrointestinal blood passes by the liver before it reaches the general systemic circulation. The liver may detoxify and eliminate certain compounds from the upcoming blood, in order to prevent their distribution in the systemic blood circulation. Since the liver is the organ where the metabolism of most ingested compounds, these agents, as well as their metabolites may be excreted into the bile without entering the bloodstream again to be excreted by kidneys. A xenobiotic could be excreted by cells of the liver into the bile, which transfer it to the small intestine. If the chemical or its metabolites are favourable for intestinal reabsorption, then they will be reabsorbed and an enterohepatic cycle may begin. In the latter, the biliary secretion and reabsorption by the intestines keep occurring until the renal excretion eliminates the chemical from the body. The biliary excretion has a major role in the elimination of cations, anions, and non-ionized molecules having both polar and lipophilic groups. Xenobiotics reach the bile mainly through their active secretion, simple diffusion may occur if the chemical is lipid-soluble and can pass through parenchymal cells into the bile. However, the latter would most likely be reabsorbed by the intestine (Gupta, 2016).

■ **Gastrointestinal tract excretion**

Numerous chemicals are excreted in the feces, mainly when the chemical: was not absorbed after ingestion, excreted into the bile, or its excretion route is the gastrointestinal tract. In favourable concentration gradients, some ionized xenobiotics may be excreted into

the gut through a passive diffusion process. However, organic acids and weak bases which are scarcely ionized at pH=1 are not excreted into the gut (Gupta, 2016).

- **Expired Air excretion**

    Volatile compounds entering the lungs could be excreted unchanged during expiration., such as fluorobenzene. Volatile agents/gases with low blood/gas solubility such as benzene are excreted rapidly, however, agents with high blood/gas solubility, are excreted in a very slow rate by expiration (Gupta, 2016).

- **Excretion through sweat, and milk**

    Generally, a scarce amount of chemicals is excreted through sweat. The latter, is an acknowledged excretory route for toxic elements. Arsenic, cadmium, lead and mercury can be excreted in remarkable quantities through the skin, with rates matching those of the kidneys, and sometimes exceeding the kidneys' excretion rate in 24 hours (Sears et al., 2012).

Certain xenobiotics and their metabolites are partially excreted in the milk of lactating mammals. They are excreted by simple diffusion, due to the acidic nature of milk (pH 6.5-6.7) comparing to plasma. Basic agents may be present in high concentrations, and the opposite is expected with acidic compounds, such as: DDT (Gupta, 2016),

II.4.5. Factors influencing toxicity

A xenobiotic's toxic capacity is dependent on many factors:

1. Bioavailability is described as "The rate and extent to which the active constituent or active moiety of a drug is absorbed from a drug product and reaches the circulation"(Page and Maddison, 2008). For a toxicant to be toxic, it must be bioavailable within the body (Hill, 2010). Bioavailability is influenced by many factors, including physical and chemical properties. For example: Elemental mercury is volatile at room temperature. If it was inhaled, the majority of it may be absorbed through alveoli into the bloodstream.

2. The absorption route through which they exert their toxicity. While some are only toxic by one route, others could be toxic through many routes. For example: formaldehyde exerts its carcinogenic effect only through inhalation. In contrast, arsenic exerts its toxicity through the three routes: inhalation, ingestion, and skin absorption (Hill, 2010).

3. Storage: After absorption of the xenobiotic into the blood, a part of it can be stored for short, or long periods of time. Provided that the chemical agent is bound, it does not induce its toxic effects (Hill, 2010). That is due to the fact that it would not be bioavailable. For example: certain heavy metals that are found in chronically exposed animals to pollutants, could be found bounded to a protein "metallothionein" in the kidneys. Making the heavy metal not bioavailable.

II.5. Fate of non-excreted pollutants

II.5.1. Bioaccumulation

"Bioaccumulation occurs when an organism absorbs a toxic substance at a rate greater than that at which the substance is eliminated." (Michalak and Chojnacka, 2014). A chemical is considered to be bioaccumulated when its concentration within an animal exceeds the chemical's concentration in the environment. For example: lead (Pb) bioaccumulate in bones, xenobiotic heavy metals (such as cadmium) bioaccumulate in the liver, kidneys, and other soft tissues bonded to certain proteins (Hill, 2010).

II.5.2. Biomagnification

Biomagnification results from the increase on a chemical agent's concentration along the food chain, as a consequence of bioaccumulation and bio-transfer (Kodavanti et al., 2014). Additionally, the concentration of the chemical agent in tissues of organisms at a trophic level is greater than the concentration in the tissues of those at a lower trophic level (Kodavanti et al., 2014). In other words, "a pollutant reaches progressively higher concentrations as it moves through the food web it is said to bio-magnify" (Hill, 2010).

III. Heavy metals and metalloids' health impact

In medicine, the term "heavy metals" encompasses all toxic metals, including those that are light. Thus, 'heavy metal poisoning' includes poisoning caused by heavy metals, semimetals, such as Arsenic, as well as, lighter metals such as aluminium. However, bismuth is excluded due to its low toxicity (WHO, 2011).

Heavy metals can be categorized into two groups: essential and non-essential heavy metals. Essential ones are considered micronutrients, such as iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), and nickel (Ni) (Gall et al., 2015). Such heavy metals are required for

different physiological and biochemical processes in the human body, However, their presence above or under their optimal thresholds could result in deficiencies and acute/chronic toxicities (Azeh Engwa et al., 2019). The second category consists of non-essential heavy metals; such as aluminium (Al), arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg). These are not required for biological processes, and rather rapidly induce toxicity (Gall et al., 2015). Contamination by heavy metals starts by the absorption of the contaminant through several routes. Some of them, such as "lead (Pb), cadmium (Cd), manganese (Mn), and arsenic (As)" can be absorbed into the body through the gastrointestinal tract (Azeh Engwa et al., 2019). Others can enter through inhalation, and skin absorption.

Essential heavy metals are only toxic above the highest thresholds, while the non-essential ones are considered toxic xenobiotics. As a result, the body maintains metal homeostasis within physiological norms, and employ metal detoxification processes to achieve it (Tamás et al., 2014). A heavy metal's toxicity is dependent on its physical and chemical characteristics, as well as its ligand affinity. Cadmium and mercury have affinity for sulfur as a ligand. Manganese, arsenic, and selenium have affinity for oxygen in their highly oxidized state, however, in their low oxidized state they have affinity for sulfur ligand. Lead, iron, cobalt, nickel, copper, and zinc have affinity for oxygen, sulfur, or nitrogen as ligands (Tamás et al., 2014). Due to their physicochemical affinities, heavy metals interact with different components of the body. When they persist in the body, they are stored and start bioaccumulating in tissues. Then, the organism will start using them as substitutes of essential elements. For example, lead can be used as a substitute for calcium, zinc could be substituted by cadmium, while the majority of trace elements could be substituted by aluminium. Additionally, stored heavy metals interfere with metabolism and create oxidative stress, which could impair the functioning of essential enzymes, and increase the risk of infection due to the affection of carbohydrate, lipid and protein metabolism.(Rehman et al., 2018).

In cells, heavy metals are known to: cause oxidative stress by inducing ROS; alter DNA or impair its repair mechanisms; impede nutrient uptake and membrane function: and disrupt protein functioning and activity. Molecularly, it is agreed that the main targets od heavy metals are proteins. They interact with them through different ways, they could: bind to free thiol groups or other functional ones; dislocate important metal ions in metalloproteins; or carry the catalysis of amino acid side chains oxidation (Tamás et al., 2014).

Various carcinogenic pathways associated to heavy metals exposure have been identified, in research. Carcinogenic and mutagenic effects have been associated to the oxidative stress that is induced by heavy metals. Metal ions such as nickel, cobalt, and arsenic carry redox reactions in the body, and generate free radicals. The produced free radicals induce oxidative damage (affecting DNA, and proteins), activate redox-sensitive transcription factors, interfere with DNA repair, and act as mitotic signals (Fu and Xi, 2020).

III.1. Toxicity mechanisms of heavy metals

III.1.1. Oxidative stress induction and biomolecules oxidation

Oxidative stress can be described as "An imbalance between the production of free radicals and the antioxidant system, which is in charge of keeping the homeostasis of the organism" (Casas-Grajales and Muriel, 2017). When the metal-ion homeostasis is disrupted, metals could bind to protein sites that are not designed for them. This process could induce oxidative damage to these proteins and macromolecules deterioration (Jomova and Valko, 2011). Certain metals are described to generate free radicals which could lead to oxidative stress and damage cells. Free radical generation is specific to potential heavy metal (Azeh Engwa et al., 2019).

III.1.1.1. Copper

Copper is an essential heavy metal, and is a cofactor of a number of enzymes participating in redox reactions. These enzymes include: cytochrome C, oxidase, and ascorbate oxidase. This metal is absorbed through the small intestine, then, it is bound to serum albumin or histidine and transported through the bloodstream to be either delivered to tissues, or stored in the liver. Copper is taken into hepatocytes through the high-affinity human copper transporter (hCtr1) on their plasma membrane. Inside hepatocytes, copper is transported to: metallothionein pool, or to the mitochondria for the incorporation to cytochrome c oxidase, or taken to emerging CuZn-SOD (superoxide dismutase), or transported to Wilson disease P-type ATP-ase in the trans-golgi network to be incorporated to ceruloplasmin (contains 95% of serum copper). Copper stored in the liver is mainly excreted through the biliary excretion route. (Jomova and Valko, 2011).

Copper ions have been known to participate in the process of ROS production, because cupric (Cu^{2+}) and cuprous (Cu^{1+}) have the ability to participate in redox reactions. In the

presence of biological reductants (eg. GSH, ascorbic acid), or superoxide anion radicals, the cupric ion (Cu^{2+}) may be reduced to cuprous ion (Cu^{1+}). The latter has the ability of catalysing the decomposition of hydrogen peroxide (H_2O_2) to produce reactive hydroxyl radicals ($OH\cdot$) (Azeh Engwa et al., 2019) , via the Fenton reaction (Jomova and Valko, 2011):

$$Cu^{2+} + O_2^{-}\bullet \rightarrow Cu^{1+} + O_2$$

$$Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + \bullet OH + OH^{-} \text{ (fenton reaction)}$$
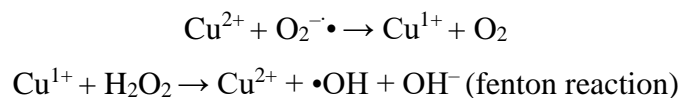
The resulting hydroxyl radical has the ability of reacting with any nearby biomolecules. Copper has been reported to have the ability to induce DNA strand breaks and the oxidation of bases through oxygen free radicals. In vivo and in vitro studies have shown LDL oxidation induction by copper. (Azeh Engwa et al., 2019).

Studies have suggested that copper can activate cellular transcription through both metal, and oxidative stress mediations. While metal induced mediation of cell signalling is still not well defined, copper-induced oxidative stress can lead to lipid peroxidation, consequently, an increase in signalling molecule HNE occurs. The latter, acts as a secondary messenger and could increase phosphorylation and activation rates of certain MAPK pathway components, namely: c-Jun N terminal kinase, stress-activated protein kinase SAPK, and P38 pathways. HNE presence is linked to an increase in AP-1 protein activity, as well as, the activation of PKC (Jomova and Valko, 2011).

III.1.1.2. Cobalt

4% of vitamin B12 is made of cobalt, which proves that cobalt is an essential metal for the body. However, studies have shown that cobalt interferes with DNA repair processes, and could directly cause DNA alteration, DNA-protein crosslinks, aneuploidy, and exchange of sister-chromatid (Jomova and Valko, 2011).

Cobalt mediated free radical generation is linked to its toxicity and carcinogenicity. Especially (Co^{2+}) which been demonstrated to give rise to superoxide radicals from the decomposition of H_2O_2 (Azeh Engwa et al., 2019), according to the reaction:

$$Co^{2+} + O_2 \rightarrow Co^{+} + O_2^{\bullet -} \rightarrow Co^{+} - OO\bullet$$
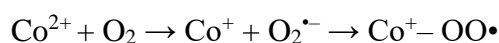
The overconsumption of cobalt >5mg/day, could lead to abnormal thyroid functioning, polycythemia, and erythropoiesis along a rise in erythropoietin production. Its toxic effects affect mostly the lungs, and may lead to asthma, pneumonia, and wheezing (Jomova and Valko, 2011).

III.1.1.3. Arsenic

The most frequent oxidation forms of arsenic are: As^{+5} (arsenate), As^{+3} (arsenite), and As^{-3} (arsenide), in these forms the semi-metal is capable of forming both organic and inorganic compounds in both the environment and the human body. Arsenic is termed "Organic Arsenic" when it is linked with hydrogen and carbon, conversely, when it is linked to inorganic elements, such as: oxygen, sulfur, and chlorine, it is termed 'Inorganic arsenic". Arsenites (AsO_2^-) and arsenates (AsO_4^{3-}) are prevalent in water, soil, or food. (Jomova and Valko, 2011).

After ingestion, inorganic arsenic undergoes metabolism involving reduction, followed by oxidative methylation to form pentavalent organic metabolites. Arsenic's organic and inorganic metabolites may be found in trivalent, or pentapriced oxidation states. Sodium arsenate, or arsenic dioxide in triaval compounds interacts with sulfur groups, which could lead to the inhibition of numerous enzymes. Methyl oxide and glutathione concosering (Fu and Xi, 2020).

The porposed metabolic pathway of Arsenic in humans is "oxidative methylation, and glutathione conjugation". Inorganic (As^{+5}) is known to be reduced to ($As^{+3)}$, which is necessary for methylation in humans. Inorganic As^{+3} undergoes a methylation to form methylarsonic acid (MMA) and dimethylarsinic acid (DMA) by interchangeably reducing pentavalent As to trivalent As (Mochizuki, 2019) (Fig.7).
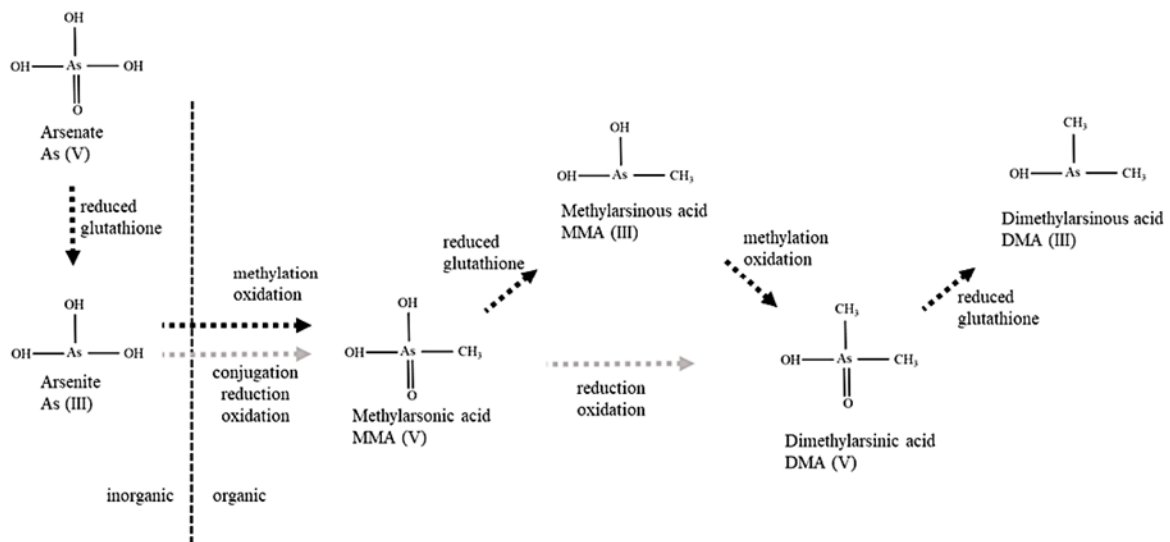
Figure 7. Proposed pathway of arsenic metabolism: conversion of inorganic arsenic into organic (Mochizuki, 2019).

Arsenic toxicity could affect the majority of organs, inorganic arsenic is known to be more toxic than methylated organic arsenic. Trivalent forms of arsenic are the most toxic and interact with protein thiol groups. Thus, inhibiting numerous cellular enzymes, mainly by binding to sulphydryl groups. Moreover, arsenic inhibits gluconeogenesis, fatty acid oxidation, acetyl CoA production, and cellular glucose uptake.

The pentavalent inorganic arsenic presents less toxicity; however, it is highly similar to inorganic phosphate. As a result, it substitutes phosphate in glycolytic and cellular respiration processes. Due to this substitution, a loss of high energy ATP phosphate bond due to the preferential formation of ADP-arsenate, leading to the uncoupling of oxidative phosphorylation. Arsenic has been reported to generate free radicals, including: singlet oxygen ($1O_2$), nitric oxide (NO), superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO), radicals of dimethylarsinic peroxyl$((CH_3)_2AsOO)$, and dimethylarsinic radicals $((CH_3)_2As)$. The oxidative stress generative mechanism of free radicals is still not well-defined.

The bioavailability of inorganic arsenic is between 60% and 87%, Most of it is excreted in urine and bile (Mochizuki, 2019).

III.1.2. Carcinogenesis

Certain heavy metals are known to have carcinogenic effects, because they target numerous cellular regulatory proteins that take part in apoptosis, regulation of cell cycle, DNA repair, DNA methylation, cell differentiation, and cellular growth. Their carcinogenicity has been linked to the activation of transcription factors that are redox-sensitive through electron recycling by antioxidants. Examples of transcription factors include AP-1, Nf-κB, and p53, these factors regulate the expression of protective genes which induce apoptosis, block the proliferation of altered cells, repair damaged DNA, and enhance the immune system's performance. The inactivation of p53 permit uncontrolled cellular division, consequently, most human cancers have been associated the disruption of p53 gene expression. AP-1 and NF-κB family of transcription factors participate in both of cellular proliferation and apoptosis, as well as, the regulation of p53. Heavy metals generated oxidative stress inside cells could selectively activate these transcription factors, which suggest that cell proliferation and death may be influenced by the exposure to heavy metals of high carcinogenic potential (Azeh Engwa et al., 2019).

III.1.2.1. Arsenic

It has been reported that liver, skin, prostate and Kupffer cell cancers were associated with arsenic poisoning. Arsenic carcinogenicity relies on a set of mechanisms, including the induction of epigenetic alterations, DNA maintenance system damage, and generation of reactive oxygen species. The main epigenetic changes induced by arsenic are: DNA and microRNA methylation, and histone damage, these alterations have been reported to own the potential of causing malignant growths. In vitro studies have indicated that arsenic changes the expression profile of p53 protein, which consequently affects the proteins that are regulated by p53, such as p21 (CDK inhibitor 1). This semi-metal has also been demonstrated to promote genotoxicity in human and mice leucocytes, moreover, methylated arsenic inhibits DNA repair and generate free radicals in the liver and spleen as products of metabolism. Additionally, its ability to bind to DNA-binding proteins (such as methyl-transferases) along with the disruption of DNA repair mechanisms magnifies the risk of carcinogenesis (Azeh Engwa et al., 2019).

III.1.2.2. Lead

Lead has been reported to impair DNA repair system, as well as, cells tumor regulatory genes through ROS production. Evidence based studies have shown that lead generated ROS are crucial in the chromosomal structure disruption and sequence alteration. Moreover, lead has the ability to disrupt cellular transcription by substituting zinc in certain transcriptional regulatory proteins (Azeh Engwa et al., 2019).

III.1.2.3. Mercury

This metal's carcinogenic ability has been suggested to occur through the production of ROS, and the induction of oxidative stress leading to biomolecules damage. Mercury has been reported to disrupt DNA molecular structure, as well as, DNA repair and preservation mechanisms (Azeh Engwa et al., 2019).

III.1.2.4. Nickel

Some of nickel carcinogenic mechanisms are: influencing the regulation of transcription factors, generation of free radicals, and controlling the expression of certain genes. Nickel participates in regulating the expression of certain long non-coding RNAs, particular mRNA, and microRNAs. This metal can help DNA methylation of MEG3 gene's (maternally expressed gene 3) promoter and cause its downregulation, thus, upregulating hypoxia-

inducible-factor-1α, which are two proteins that are known to have a role in carcinogenesis. Moreover, nickel has the ability to generate free radicals which favours carcinogenesis (Azeh Engwa et al., 2019).

III.1.2.5. Cadmium

This metal has been identified to promote apoptosis, DNA methylation, oxidative stress, and DNA damage (Azeh Engwa et al., 2019).

III.1.2.6. Copper

Literature has shown that serum and tumour tissue copper rates in cancer patients are substantially higher than those of healthy subjects. High copper levels are reported to be directly related to cancer progression. Certainly, that this metal is known to promote oxidative stress, and inflammation. Copper has been identified to stimulate angiogenesis in an animal embryonic model, additionally, angiogenic cytokines and growth factors, such as IL-1,6, b-FGF, TNF-α, and VEGF are eliminated following copper removal (Azeh Engwa et al., 2019). Due to its powerful redox actions, copper is implicated in different aspects of cell signalling, such as the modulation of membrane receptor-ligand interactions, kinase and associated phosphatase, as well as, the control of gene expression in the nucleus. The loss of copper homeostasis, either through genetic mutation, ageing, or environmental factors, leads to severe pathological consequences and contributes majorly to cancer, inflammation and neurodegeneration (Grubman and White, 2014).

III.1.3. Neurotoxicity

Certain heavy metals can affect the nervous system and induce neurological toxicity, such as lead, and manganese.

III.1.3.1. Lead

Lead can exert its toxic effects on the nervous system through three mechanisms: (a)by weakening learning and memory capacities through the inhibition of N-methyl-d-aspartate receptor (NMDAR), and blocking neuro-transmission by impeding the liberation of neurotransmitters, (b) by causing the blockage of voltage-gated calcium channels (VGCCs), (c) by reducing expression levels of brain-derived neurotrophic factor (BDNF) (Azeh Engwa et al., 2019).

III.1.3.2. Manganese

This metal accumulates in neuron's mitochondria, astrocytes, and oligodendrocytes and alter ATP synthesis, by blocking F0 ATP synthase, or NADH dehydrogenase (complex 1) of the mitochondrial respiration chain. Moreover, manganese is known to inhibit ATP synthesis in neuron mitochondria, either at glutamate/aspartate exchanger, or succinate dehydrogenase (complex II). The intracellular ATP production decrease, and generated free radicals increase oxidative stress, thus increasing manganese cellular toxicity. Additionally, dopamine can be oxidized by manganese, and reacts with quinone species, which distorts the dopaminergic system, leading to deficits, and dopaminergic toxicity, because reactive dopamine species react with dopamine transporters (DAT1) (Azeh Engwa et al., 2019).

III.1.3.3. Aluminium

Aluminium can affect phosphoinositide second messenger-producing process (responsible for calcium levels in cells), thus, modulating calcium concentrations. It also forms strong irreversible bounds with large proteins, and could inhibit neuronal microtubule formation (Keith et al., 2002).

III.1.4. Clinical manifestation of toxicity

Tab.3 presents a summary of some toxic heavy metals, their exposure routes, sources, and health adverse effects.

Table 3. Certain heavy metals exposure routes, main sources, and associated health adverse effects. Adapted from (Pratush et al., 2018; Rehman et al., 2018)

Metal	Exposure route	Major sources	Health impact
Arsenic	Ingestion via drinking water, food, smoking, occupational	Smelting, fossil fuel burning, agricultural pesticides, industrial waste	Arsenicosis, psychological effects, decreased mental performance, hypertension, cardiovascular disease risk, carotid atherosclerosis and diabetes mellitus, lung cancer, carcinogenesis
Cadmium	Ingestion via drinking water and food like fish	Mining, fossil fuel burning, manufacturing of lead—acid batteries, oxide synthesis forv paint, and pigment	Neurotoxic effects on intelligence, decreased memory, hemolytic anaemia, CVS diseases, reproductive toxicity, lung cancer, bladder cancer

Nickel	Ingestion through drinking water and food, inhalation exposure, ccupational exposure	Chemical industries, food processing industries, forest fires, volcanic emissions, incineration of waste, combustion of coal	Allergic contact dermatitis, oral hypersensitivity and risk of gingival hyperplasia, oral cancer, skin cancer, lung cancer, asthma, bronchitis, reproductive toxicity, carcinogenesis
Lead	Water ingestion, paint, soil	Mining, fossil fuel burning, manufacturing of lead—acid batteries, oxide synthesis forv paint, and pigments	Neurotoxic effects on intelligence, decreased memory, hemolytic anaemia, CVS diseases, reproductive toxicity, lung cancer, bladder cancer
Copper	Ingestion	Electroplating, metal alloys, domestic and industrial waste, mining waste, pesticides	Anemia and other toxicity effect includes indirectly through interaction with other nutrient
Zinc	Ingestion	Metal alloys, pigments, electroplating, industrial waste, pipelines	Abdominal pain, nausea, vomiting and diarrhea, irritability, leathery, anemia

IV. Lizards as an animal model for environmental pollution and toxicity assessment

IV.1. Fundamental characteristics of desert reptiles

Deserts are known to be one of the hardest environments for living organisms, mainly due to the high temperatures and the unavailability of water. Reptiles can be considered the most adapted vertebrates to desertic environments, due to their unique characteristics (Bradshaw, 1997). They prevail in hyper-arid areas in comparison to other vertebrates, owing to their physiological and behavioural attributes which make them particularly suited to resource-limited environments (Bradshaw, 1997). These basic characteristic physiological and behavioural attributes are (Bradshaw, 1997):

▪ Low metabolism rates compared to birds and mammals, which enable them to scarce rates of resource utilisation and lead to economic water management.

▪ Ectothermy: through the reliance on external heat sources, reptiles have an inherent capacity to thermoregulate by behavioural approaches (rather than energy consuming physiological ones).

▪ Faculty of excreting protein digestion products (nitrogenous waste) in a highly insoluble uric acid form, therefore, preserving the water.

- Ability to endure pronounced perturbations of internal homeostasis, for long periods of time, which could be lethal to mammals and birds.
- Using avoidant behavioural strategies for survival, during periods of disadvantageous continued activity.

Reptiles are differ from mammals and birds, in two main physiological aspects: their ectothermy, and their reliance on an anaerobic metabolism to generate energy for high or persisting activity (Bradshaw, 1997). Ectotherms are animals that regulate their body temperature through external sources, such as: sunlight and heated rock surfaces. Reptiles are ectotherms, as a result, they use low rates of energy (Bradshaw, 1997).

IV.2. Lizards in research

Among the 11440 reptile species, 6972 are lizards (P.Uetz, 2021). Numerous aquatic and terrestrial species have been used to assess environmental pollution and its related impact on the eco-systems. (Silva et al., 2020) Lizard populations among these species are especially threatened by environmental degradation, because they are mostly associated with specific habitats (Campbell, 2000; Silva et al., 2020)

Organisms can be considered evaluation models for environmental pollution if they: exhibit reactivity to environmental contamination; are abundant in the targeted area; have low migratory rate; reduced displacements. Which are characteristics the majority of lizards have, (Silva et al., 2020). Lizards are ectothermic and have a low metabolic rate, which makes them more sensitive than mammals and birds to the effects of chemicals—with the exception of fish, which appear to be more sensitive—. Due to their ectothermic nature; low-rate metabolism; and their simple enzymatic systems, lizards have a weak capacity to rapidly detoxify absorbed, inhaled, or ingested chemicals (Campbell, 2000). Thus, their recovery could take longer than other non-reptilian species (Hall, 1980).

Some of the most relevant sources of exposure to chemicals are a contaminated food chain, and dermal exposure to chemicals (Silva et al., 2020). Lower organisms on the food chain might bioaccumulate contaminants of organic or metallic nature, thus, creating a biomagnification cycle within organisms higher on the food chain. On the other hand, dermal contamination is considered an important route for pesticides and airborne pollutants (Silva et al., 2020)

Despite the scarcity of studies utilizing reptiles from the Squamata group for environmental pollution assessment, ecotoxicological studies have been improved to better demonstrate environmental pollution effects on lizards (Campbell, 2000). This is due to the fact that squamates have a high potential as bioindicator organisms in environmental risk evaluation, and have shown excellent examples in ecotoxicological studies (Campbell, 2000). Reptiles can have a direct exposure to pesticides through many possible ways, such as "(a) ingestion of contaminated food; (b) accidental or deliberate ingestion of soil; (c) inhalation; (d) maternal transfer to eggs/ young; (e) dermal exposure; (f) absorption by eggs of contaminants from surrounding environments."(Amaral et al., 2012)

Adult reptiles are secondary and tertiary predators in the food chain, making them at risk of bioaccumulating persistent environmental pollutants, particularly organochlorine pesticides. Reptiles are efficient for heavy metal contamination biomonitoring. They bioaccumulate and biomagnify chemicals to equal or higher levels than those reported to be found in birds and mammals (Campbell, 2000). Additionally, correlations were found between heavy metal contaminated preys and the bioaccumulation of those metals in lizards (Campbell, 2000). Lizards are essential components of food chains, thus, environmental contaminants could reach humans through bioaccumulation routes comprising lizards (Campbell, 2000).

CHAPTER II

MATERIALS AND METHODS

I. Location of the experimental work

This experimental work was achieved in three laboratories. Animal handling and histopathological analyses were carried in the histotechnology laboratory of biotechnologies department, faculty of nature and life sciences, university Blida 1. The samples digestion procedure was carried in the research laboratory of Biotechnology of Plant Productions, University Blida 1. The Atomic absorption spectrometry analysis was achieved in the Center of Scientific research and Techniques in physicochemical Analyses (CRAPC) Bousmail, Tipaza. During the period of May – June 2021.

II. Data on the biological material

IV.3.1. Taxonomy

Tab.4 represents *S.scincus'* detailed taxonomy.

Table 4. Classification of the *S.scincus* in the animal kingdom (Grandison, 1959)

Kingdom	Animalia
Phylum	Chordata
Class	Reptilia
Order	Squamata
Family	Scincidae
Genus	Scincus (Laurenti 1768)
Species	*Scincus scincus* (Linnaeus, 1758)

IV.3.2. Bio-ecology and biological characteristics

In deserts, lizards constitute a substantial part of the vertebrate density and diversity, with the majority of them (lizards) being insectivorous. Numerous skink species (Scincidae) have been reported to reach massive densities in arid regions of Africa and Australia (Campbell, 2000).

The common skink (*Scincus scincus*) (Linnaeus, 1758) (Fig.8) belongs to the scincidae family, and is found in the deserts of North Africa and the Middle East (Stadler et al., 2016). This specie exhibits remarkable abilities of sand diving and rapid movement through the dry sand medium over considerable distances. Moving between layers of sand is also a way through which the lizard regulates its body temperature. Certainly that the desertic environment is known to undergo substantial temperature changes (Stadler et al., 2016).
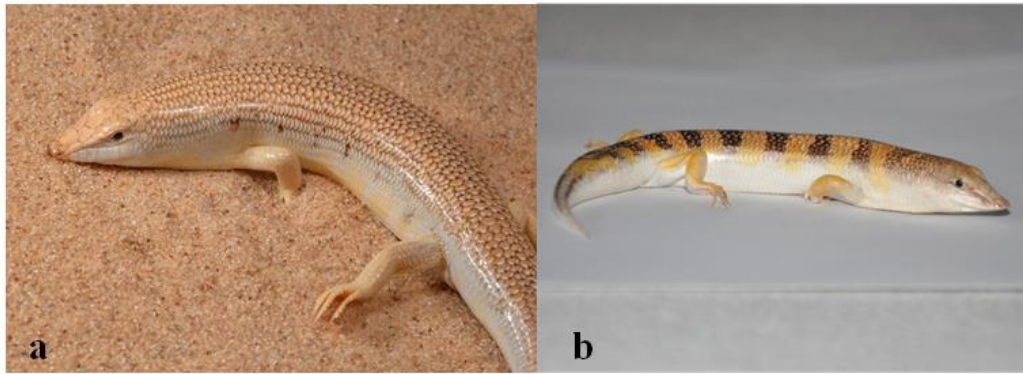
Figure 8. Photographs of (*Scincus scincus*)
(a) Female (b) Male

According to previous longevity records, the common skink's average life span may be considered to be 10 years (Ellis et al., 2011). The average adult sandfish weights around 25g and has a size of about 17cm. Some of their morphological adaptations include a wedge-shaped snout, smooth scales, concealed ear apertures, valvular nostrils, countersunk lower jaw (Šmíd et al., 2021). These diurnal species are primarily insectivorous, feeding on an arthropod diet. Most temperate species have been reported to lower their food intake during hot summer or cold months (Paray et al., 2018). Which suggests that feeding patterns of Scincus scincus change depending on seasons and climate. In a previous studies on Scincus Hemprichii and Scincus mitranus feeding habits, it was observed that the feeding activity becomes high during November, and March (Paray et al., 2018).

III. Methods

III.1. Sample collection

Seven male n=07 adult skinks weighting between 25 and 30g (fig. 9), were collected in May from El Oued Souf area (latitude: 33, 3683° North; longitude: 6.8674 East; altitude 70m), which is characterized by a hot desert climate (Köppen climate classification BWh), with hot and dry summers, mild winters, and light sporadic precipitations. Lizards were kept in the laboratory for three weeks before starting the analyses. They had *ad libitum* access to food (dates, plant aphids, and grains) and water.

Animals were anaesthetized by inhalation, using chloroform. Selected organs, namely: brain, liver, kidneys, muscles, and skin were removed by dissection under a stereomicroscope. The specimens were divided between atomic spectrometry and morphological studies.
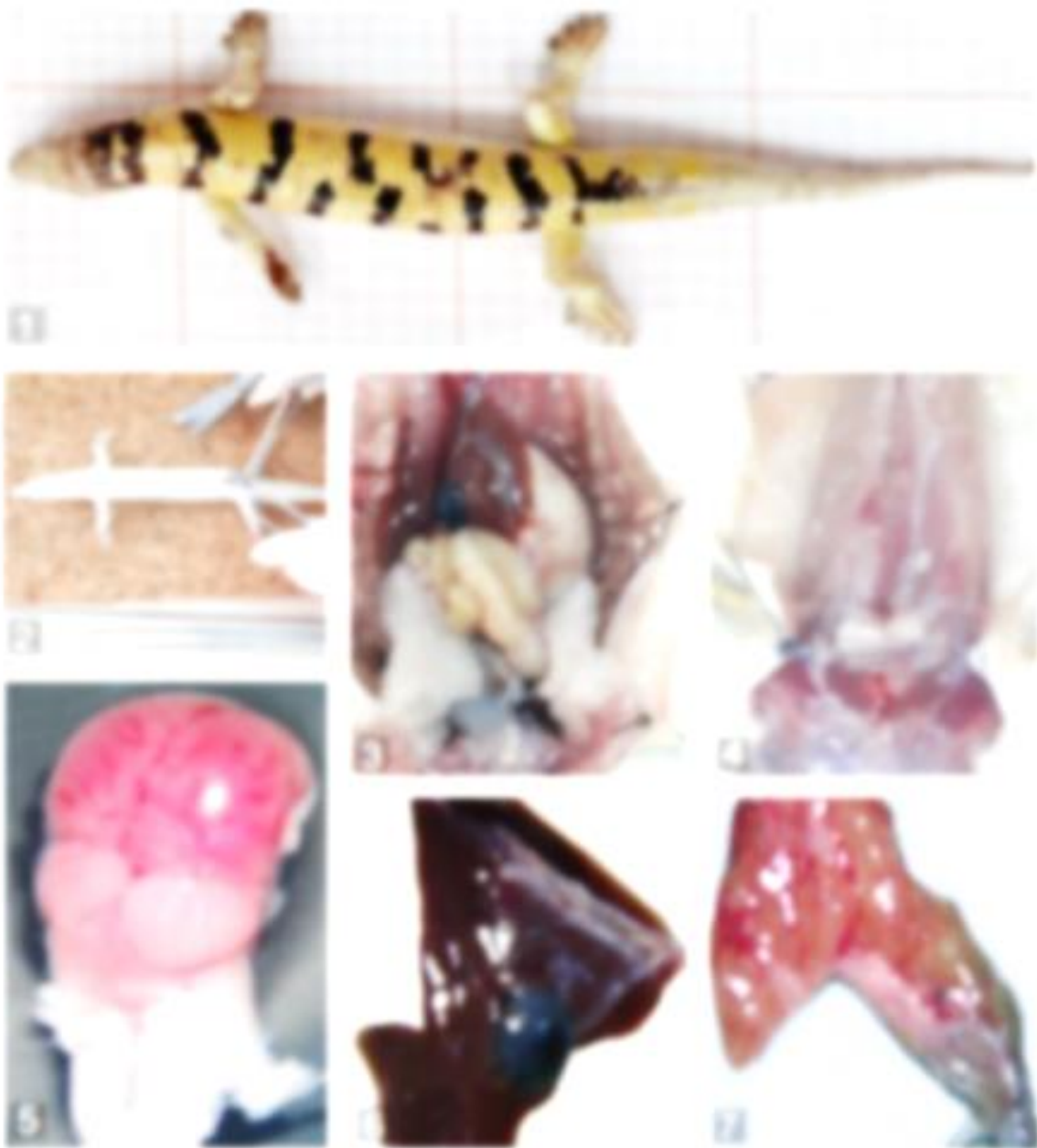
Figure 1. Tissues and organs of study interest of *...*

Male *...* is about 17.5 cm length and 1 mm width at the middle of the body. 2. Dissection of abdominal and pelvic cavities gives access to liver and kidney. The brain is obtained after opening of the skull at the cranial side. 4. Skin and muscle. 5. Brain. 6. Liver. 7. Kidney.

- For the atomic absorption spectrophotometry study, two samples were made. The first one consisted of liver and kidneys, while the second was contained muscles and skin. Subsequently, the two groups of samples (of seven lizards) were homogenized separately (liver with kidneys, and muscles with skin) using a mortar and a pestle.

- For the histopathological study, selected tissues were fixed in 4% phosphate-buffered paraformaldehyde for 72 hours (see annex), in order to prevent tissue autolysis and putrefaction of specimens.

III.2. Spectrometry of atomic absorption technique

III.2.1. Preparation of samples

The two homogenised samples of 07 lizards' (kidneys with livers and, muscles with skin) underwent chemical digestion according to (CEAEQ, 2003) The first step was to homogenize tissues mechanically using a mortar and a pestle. One 1g of each sample was weighted and put inside a heat resistant round-bottom flask, 5mL of 68% nitric acid (HNO_3) solution was added, then left to rest for 30 minutes. A sand bath was made by putting sand inside a heat resistant trough, which was then heated using an electric heater. After 30 minutes, the two flasks containing samples and nitric acid were left in the sand bath at 150°C until the solution volume reached 0.5-1mL. That was around 2 hours of heating. Afterward, 2mL of 30% Oxygenated water (H_2O_2) were added. Then, the solution was left to evaporate to half of the initial quantity, without reaching dryness that was attained around 15 minutes.

After this step, 2mL of 68% nitic acid was added, along 1mL of 38% HCl to the flasks. Then after light agitation and some gas evaporation, the round-bottomed flask was then linked to a refluxing system as described by (Labat, 2010), which does not allow evaporation of the forming gases, and heated at 150°C for one hour (Fig. 10). The flask was opened and gases are allowed to escape for a few minutes, after 30 minutes. The flask is then linked again to the refluxing system and left for another 30 minutes. One hour has ended, the flasks were put in cold water and each solution was put in 50mL polypropylen tubes. Finally, the volume of each solution was completed to 30mL with pure distilled water and agitated in order to be ready for analysis.

Figure 10. Established digestion apparatus

III.2.2. Principe and procedure of atomic absorption spectroscopy

Atomic absorption spectroscopy is used to determine the concentration of defined metals in a sample, and yields qualitative and quantitative results.

If the right amount of energy is applied to an atom, the latter will absorb that energy, and the outer electron of its orbits will be moved into a less stable configuration, and the atom becomes in an "excited state" (Fig. 11). Subsequently, due to the instability of the excited state, the atom will spontaneously and rapidly return to its ground state (stable state and decay). Thus, the electron will return to its stable orbital position, and a radiant energy of an equal magnitude to the energy initially absorbed (the applied energy) will be emitted (Fig. 11) (Beaty and Kerber, 1978).
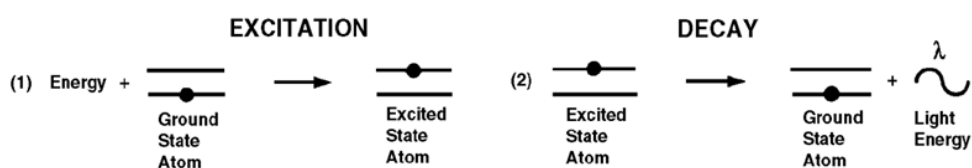
Figure 11. excitation and decay process of an atom (Beaty and Kerber, 1978).

The wavelength of the radiant energy liberated by the atom is specific to each element, because each atom has its distinct electronic orbital configuration. Large atoms could have a complex electronic structure, thus, there could be many possibilities of transition, each resulting in a unique wavelength of light (Fig. 12) (Beaty and Kerber, 1978).

Figure 12. Energy transitions (Beaty and Kerber, 1978).

In atomic absorption spectroscopy, the energy absorbed by the atom or the one that is released by it are measured for analytical ends. Quantitative atomic emission techniques rely on the detection of the intensity of emitted light at the characteristic wavelength of a known element, that is to quantify how much of an element is present in a sample (Fig. 13).
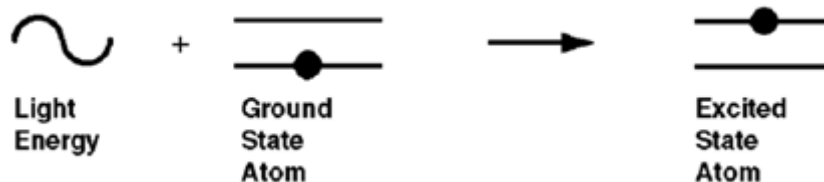
Figure 13. Emitted light absorption process (Beaty and Kerber, 1978)

In our analysis two atomic absorption spectrometers were used, both of them rely on quantitative emission techniques. The digested samples were subjected to a high energy thermal environment, in order to make atoms in an excited state, making them able to liberate light. The source of energy was a flame (240FS), or a graphite furnace (240Z). The atom cloud required is produced by providing enough thermal energy to dissociate chemical compounds of the sample into free atoms. This can be achieved by aspirating the samples into a flame; alimented by air-acetylene (2300°C), or air-nitrogen protoxide, or air-nitrogen protoxide-acetylene (3000°C); that is with a light of a hollow cathode lamp. Once the atoms cloud is formed, a light of suitable wavelength is emitted from the spectrometer lamps. Atoms in the cloud may absorb the light and become in an excited state. Then, by measuring the amount of absorbed light (through a sensor) the quantity of the targeted element could be determined (Fig. 14).
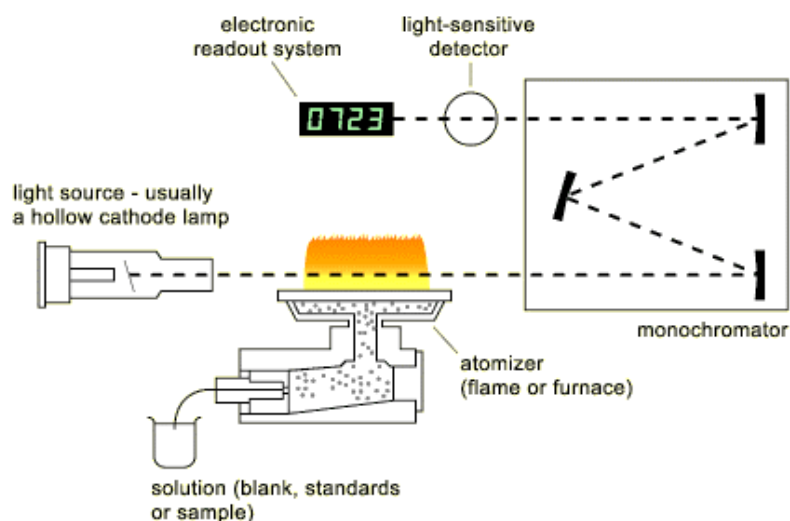
Figure 14. Flame Atomic Absorption Spectrometry process and components (Burrows, 2004a).

In the graphite furnace analysis (Fig. 15), a known volume of sample is injected into the furnace tube, the sample solution undergoes, afterwards, a multi-step temperature program. When the temperature reaches the atomization stage of the sample, the atomic absorption occurs. In this analysis, only a small volume of mineralized samples can be analysed, the maximum volume is 100µL (20µL is the recommended volume). The process was automated and carried by an autosampler (Beaty and Kerber, 1978).
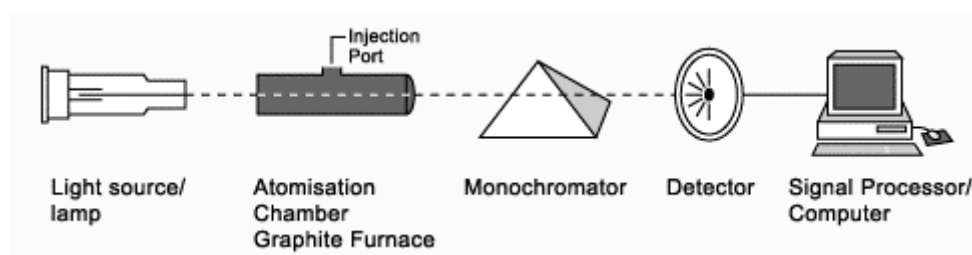
Figure 15. Graphite Furnace Atomic Spectrometer components (Burrows, 2004b)

The analysis undergoes 3 steps (Burrows, 2004b):

1. Drying: 80 – 200° C   Removes solvent from sample
2. Ashing: 350 – 1600° C   Removes organic and inorganic material
3. Atomisation: 1800 – 3000° C   Generation of free analyte atoms in light path

III.3. Histology techniques

III.3.1. Tissue processing

In order to make paraffin blocs of tissues, it is essential to let paraffin infiltrate into them. After fixation, the samples were first dehydrated by immersion in alcohol baths of increasing concentrations, that is alcohol 50%, alcohol 75%, alcohol 90%, alcohol 100%, alcohol 100% each for one (1) hour. The samples were then cleared in two (02) to three (03) bathes of xylene or toluene to remove alcohol and allow paraffin infiltration inside the tissues. After that, samples were placed into two (2) molten paraffin Baths at 60°C (Fig. 16). Finally, the specimen was embedded in paraffine molds and blocks were created.
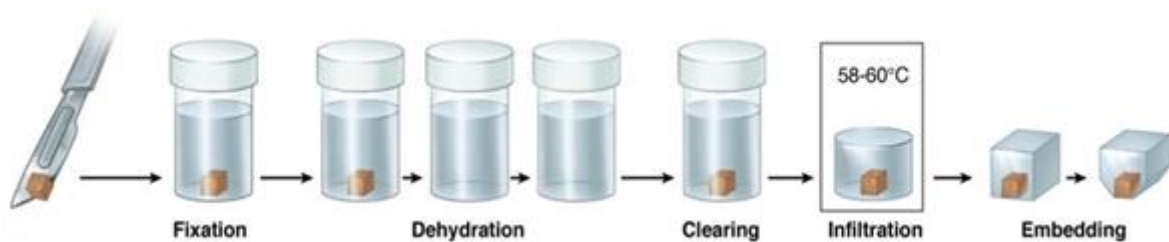
Figure 16. Tissue processing steps (Lwesya, 2019)

III.3.2. Sectioning of paraffin blocks

Before performing microtomy, gelatin-coated slides were prepared (see annex), in order to prevent paraffin sections from slipping. After embedding tissues in paraffin, solid blocks were cut into sections of 3-5 µm of thickness in a rotary microtome (Leica RM 2035).

III.3.3. Adherence of sections into slides

After sectioning, tissue ribbons were transferred into a warm bath of water (45°C), to allow paraffine sections to stretch without folding. The ribbons were picked up by placing slides under them, followed by a lifting movement. Subsequently, slides containing ribbons were placed delicately on a heating plate in order to allow the excess of paraffin to melt, and the tissue to be dried and stay well-adhered to the slide.

III.3.4. Preparation of slides for staining

Before proceeding to staining, the excess of paraffin is removed by heating slides at 60°C, and clearing in toluene then rehydrating tissues in alcohol baths of decreasing concentrations, that is 100%, 90%, 70% and 50% followed by immersion in distilled water for a few seconds (see annex).

III.3.5. Haematoxylin and Eosin Staining

- **Principle**

This staining is used to show the morphology of tissues and cells. Haematoxylin dyes are of a basic nature; thus, their charge is positive, making them attracted to acidic/basophilic structures such as regions that are rich in nucleic acids. Because these structures contain nucleic acids, which are negatively charged and acidic. Haematoxylin needs to be conjugated with a mordant (aluminium salt) in order to strengthen its positive charge. The mordant binds to the tissue structures, then, haematoxylin will bind to the mordant to form "tissue-mordant-haematoxylin" complex. This will result in the staining of nuclei and chromatin bodies in purple. On the other hand, Eosin is an acid dye which makes it eosinophilic, and its charge is negative. Therefore, it stains acidophilic structures in a red or pink colour such as the cytoplasm which is rich of basic proteins becomes pink when stained.

- **Staining procedure**

There are several classes of haematoxylin staining solutions; in the present study, we have chosen to stain our tissues with a type of alum haematoxylins, according to Carazzi, (1911) (Suvarna et al., 2019). Haematoxylin was first dissolved in glycerol overnight. Potassium alum mordant solubilised in the water was added slowly. Afterward, the solution was oxidized chemically with potassium iodate. Once the preparation is ready to use slides were immersed in the solution for 10 min, then after washing were passed in eosin (1%) bath dissolved in alcohol for 20 s (see annex).

III.3.6. Masson's Trichrome staining

- **Principle**

This staining is composed of three dyes that stain distinct structures. *First a* chromium haematoxylin dye is used to stain the nuclei. This dye is resistant to decolorization by acidic staining solutions. The second stain is the acid fuschin solution that stains acidic tissues such as muscle, cytoplasm, and collagen. A decolorizing step is necessary by using phosphomolybdic acid, this leaves the muscle cells stained in red. The third dye is aniline blue that stains the collagen along the addition of 1% acetic acid to differentiate tissue sections. The results are the appearance of nuclei in black, the collagen blue in a red background.

- **Procedure of staining**

After rinsing in running tap water, the slides were immersed in chrome haematoxylin, followed by an immersion in acid fuchsin, then a differentiation in phosphomolybdic-

phosphotungstic acid solution, until red colour is removed from collagen. Subsequently, the slides were passed in aniline bleu and after a brief rinsing in distilled water they were differentiated in 1% acetic acid, and washed with distilled water afterwards.

II.3.7. Nissl staining

- **Principle**

It is used to stain certain nervous system structures. Neurones contain a substantial amount of Nissl bodies, which are composed of rough endoplasmic reticulum. Their basophilic nature attracts acid dyes to them, such as cresyl violet. After staining, Nissl bodies appear coloured in dark blue to purple.

- **Staining procedure**

According to (Paxinos and Watson, 2005) method, the slides were deparaffinated through 5 min immersions in: Xylene (twice), 100% alcohol (twice), 95% alcohol, 70% alcohol. Followed by dipping in distilled water and staining in cresyl violet for 15-30 min. Afterwards, the slides were differentiated in water for 3-5min and then dehydrated through 70% alcohol, 95% alcohol and 100% alcohol (twice). They were then put in xylene and cover-slipped (see annex).

III.3.8. Mounting slides

In order to mount in permanent mounting medium (Eukitt), sections were dehydrated through dipping in increasing ethyl alcohol baths: 70%, 95%, 100%, followed by dips in two (02) toluene baths. A drop of the mounting medium was put afterward, the slide cover-slipped, and the medium is allowed to spread slowly.

III.3.9. Image acquisition

The sections were observed by a light microscope OPTIKA and images were captured by OPTIKAM HDMI EASY.

IV. Statistical analyses

The difference in heavy metals charges found in the different tissues of *Scincus* were evaluated by a pair comparison analysis through Wilcoxon test, consolidated by the Monte Carlo test. The differences were considered significative at 5%. Trends of heavy metals charge in the two sample groups in comparison to WHO norms, were assessed by Tenary test. Analyses were conducted using PAST software ver 3.2 (Hammer et *al*., 2001).

CHAPTER III

RESULTS

This chapter presents two aspects: The first is devoted to the research of heavy metals in different tissues of *S.scincus* (liver and kidneys & skin and muscles), while the second deals with the potential histopathological effects of heavy metals' accumulation in different tissues of the sandfish.

I. RESEARCH OF HEAVY METALS CHARGE IN TISSUES OF *S.SCINCUS*

The heavy metals research targeted 12 elements. Which were found in the animal's viscera (liver and kidneys), and in its skin and muscles.

I.1. Quantification of heavy metals in the sampled tissues

The sampled fractions (1g of biological material), after mineralization and Atomic Absorption Spectrometry analysis, have revealed the presence of diverse heavy metals quantities. The liver and kidneys contained greater quantities of copper (Cu), manganese (Mn), chrome (Cr), nickel (Ni), mercury (Hg), selenium (Se), and aluminium (Al). On the other hand, the skin and muscles have been shown to present more cadmium (Cd), zinc (Zn), arsenic (As), and lead (Pb) than those found in the skin and muscles (table 5).

Table 5. Heavy metals availability (1g of biological material)

Elements	code
Cobalt	Co
Cadmium	Cd
Copper	Cu
Manganese	Mn
Zinc	Zn
Chromium	Cr
Nickel	Ni
Mercury	Hg
Selenium	Se
Arsenic	As
Lead	Pb
Aluminium	Al

I.2. Quantification of heavy metals in tissues of one individual *S. scincus*

Results that are displayed in table 6, denote accumulated quantities of elements in one *S.scincus* individual. In order to compare accumulated quantities of metals with those recommended by WHO, we multiplied heavy metals quantities obtained from one gram of sample by 25g, which is the average weight of an S.scincus animal. The same table shows that the overall recorded quantitative data in one individual animal exceed substantially the daily recommended quantities by WHO.

Table.6: Availability of heavy metals (one lizard S.*scincus* ≈25 g)

Elements	code	WHO Recommandations (mg/J)
Cobalt	Co	0.008
Cadmium	Cd	0.035
Copper	Cu	3
Manganese	Mn	3
Zinc	Zn	40
Chromium	Cr	50
Nickel	Ni	0.162
Mercury	Hg	0.008
Selenium	Se	0.001
Arsenic	As	0.18
Lead	Pb	0.0125
Aluminium	Al	6

We resorted to the analysis per pairs of Wilcoxon and Monte Carlo, to determine the gap differences for each group of tissues. Analysis results are mentioned in table.7. As regards the kidneys and liver sample, the tests indicate the presence of a significant difference between their charge of heavy metals and the recommended amounts by WHO. In contrast, the heavy metals charge in the skin and muscles sample displays the absence of a significant difference in comparison to WHO norms.

Table 7. Comparison per pairs between the availability of heavy metals in tissues and WHO norms. (Accumulation per individual animal)

	L-K	WHO	WHO	S-M
Number of elements	12		12	
Mean	11.13	8.53	8,53	10,01
Wilcoxon Test *p(same)*	0.0413*		0.099NS	
Monte Carlo Test *p(same)*	0.0427*		0.109NS	

L-K: Liver and kidneys, S-M: Skin and muscles, WHO: World Health Organization NS: Non significative at 5%, *: Significative at 5%,

I.3. Quantification of heavy metals in tissues of five *S.scincus* according to a daily ration

According to the culinary practices of El Oued Souf region, it has been reported that S.scincus flesh is omnipresent in the Soufi household both under dried and lively forms (grilled skewers, traditional plates, and powder). We tried to see what would be the heavy metal intake of a daily ration of 5 *S.scincus*. Results are shown in table.8.

Table 8. Heavy metals availability (5 individuals of *S.scincus* ≈25 g.)

Elements	code	WHO Recommandations (mg/J)
Cobalt	Co	0.008
Cadmium	Cd	0.035
Copper	Cu	3
Manganese	Mn	3
Zinc	Zn	40
Chromium	Cr	50
Nickel	Ni	0.162
Mercury	Hg	0.008
Selenium	Se	0.001
Arsenic	As	0.18
Lead	Pb	0.0125
Aluminium	Al	6

Overall, the registered heavy metals amount in the two samples (liver and kidneys & skin and muscles) exceed the recommended limit quantities by WHO. Wilcoxon and Monte Carlo tests, point out highly significative differences between the registered quantities and WHO norms (Tab.9)

Table 9. Comparison by pair between the availability of heavy metals and WHO norms. (Accumulation per 5 individual animals)

	L-K	WHO	WHO	S-M
Number of elements	12		12	
Mean	55.65	8.53	8,53	50,08
Wilcoxon Test *p(same)*	$2.21 \times 10^{-3**}$		$2.27 \times 10^{-3**}$	
Monte Carlo Test *p(same)*	$5.2 \times 10^{-4**}$		$5.1 \times 10^{-4**}$	

L-K: Liver and kidneys, S-M: Skin and muscles, WHO: World
Health Organization ** : Significative at 0.1%,

I.4. Heavy metals ratio of *S.scincus* tissues in comparison to World's Health Organization norms

In a trend visualization mindset of heavy metals' burden in the two groups of tissues in relation to WHO norms, we resorted to the Tenary analysis. The latter, allows the prediction of accumulation trends through registered heavy metal values. The figure.17 relative to the heavy metals burden on one individual *S.scincus*, shows that manganese, copper, zinc, and cobalt, are signalled to be present in tolerable quantities. Additionally, chromium displays a remarkable value, but is still under the tolerance line.

In contrast, liver and kidneys contain especially excessive quantities of mercury, and selenium, with lesser degrees of aluminium, and nickel. While the skin and muscles are individualized with substantial amounts of arsenic, followed by lead, and cadmium.
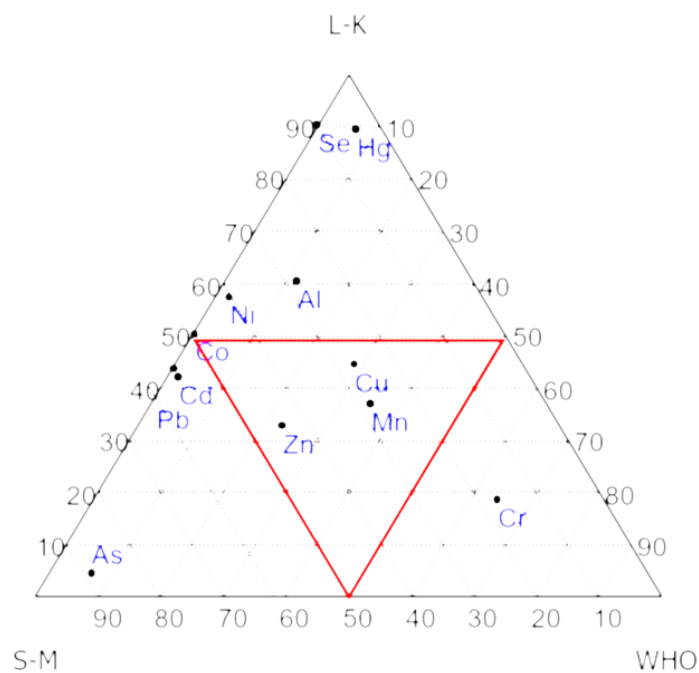
Figure 17. Trends of heavy metals charge in a nutritional ration of one individual *S.scincus*

L-K: Liver and kidneys, S-M: Skin and muscles, WHO: World Health Organization

In the daily nutritional ration estimation, the Ternary test shows that the sole tolerable element is chromium. While the skin and muscles, in addition to the high rate of arsenic, lead and cadmium, are fortified by a substantial amount of zinc (which exceeds the recommended daily intake limits Fig.18). This trend indicates the severity of consuming 5 individual skinks in one day, especially their skin and muscles.
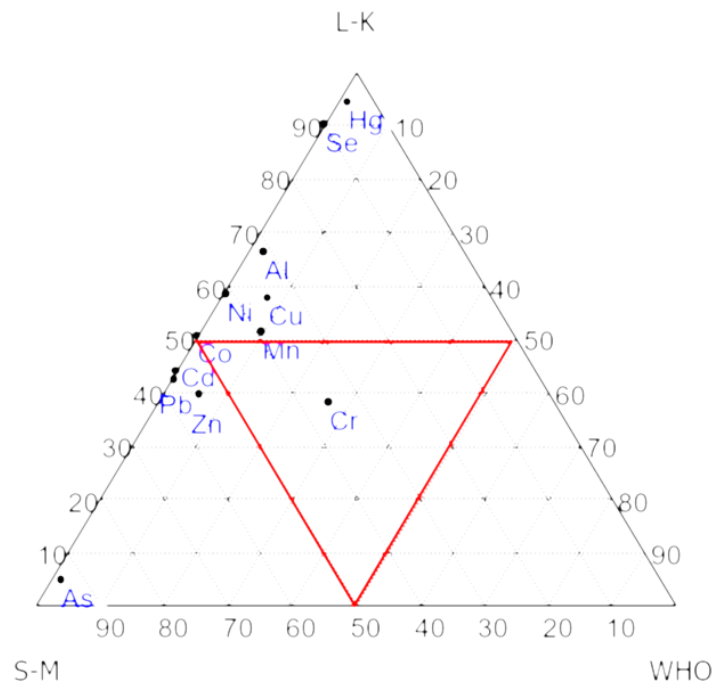
Figure 18. Trends of heavy metals charge in a nutritional ration of five individual *S.scincus*

L-K: Liver and kidneys, S-M: Skin and muscles, WHO: World Health Organization
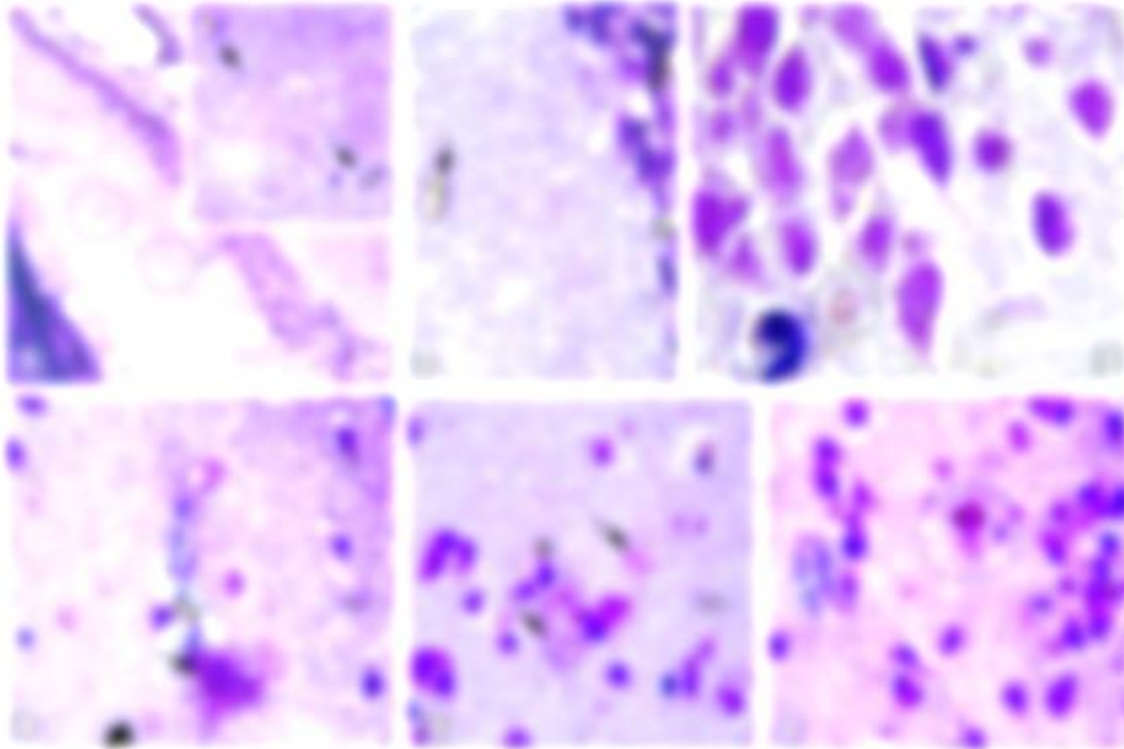
Figure 19. Photomicrographs demonstrating different non geometrical shaped plaques in brain tissue

1and 2. Circules in 1 indicate plaques found in brain tissue, magnified in inset and in 2 (arrows).
3. Plaques are found near a set of neurones (N). 4. plaque of diffused materiel (arrow) sometimes found near capillaries (Cp).
5. An other type is found between other types of neurones (N). 6. plaque with chromogene materiel is observed between sets of neurones (N). Nissl staining.
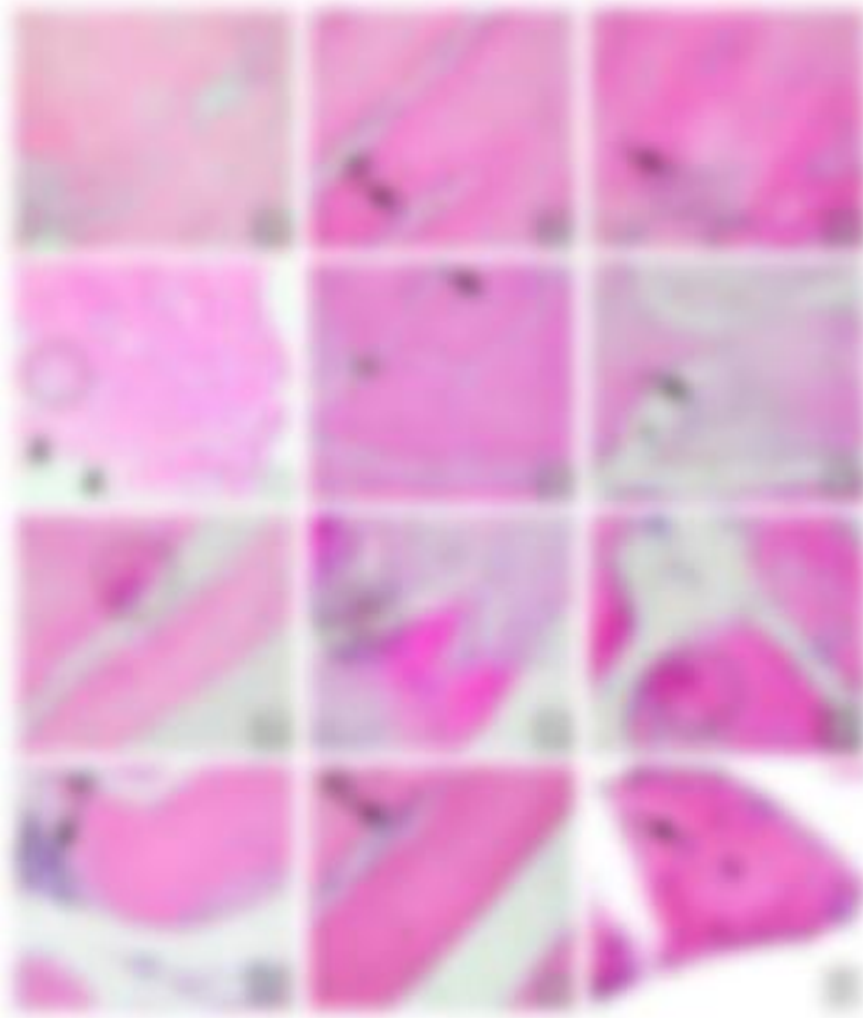
Figure 20. Photomicrographe demonstrating histopathologic manifestation in muscle

1. 2. General aspect of muscle fibers. 3. a set of dark filiforme structures are found (arrow)
4, 5, 6. Circule indicate streched and rifted fibers,frequently found (light arrowheads),
comparing to others (darck arrowheads).
 Note presence of rifts in fibers (arrow in 5 and 6), and clefts in intercaled disks (asterisk in 6).
7, 8, 9. Different types of plaques are observed (circules)
10, 11. Satellite cells are present in abundance at the interstitiel part of parenchyma (arrows).
12. Mulitinuclei are observed in the cytoplasme of muscular fibres . sC: satellite cells.
Hematoxylin & Eosine staining.
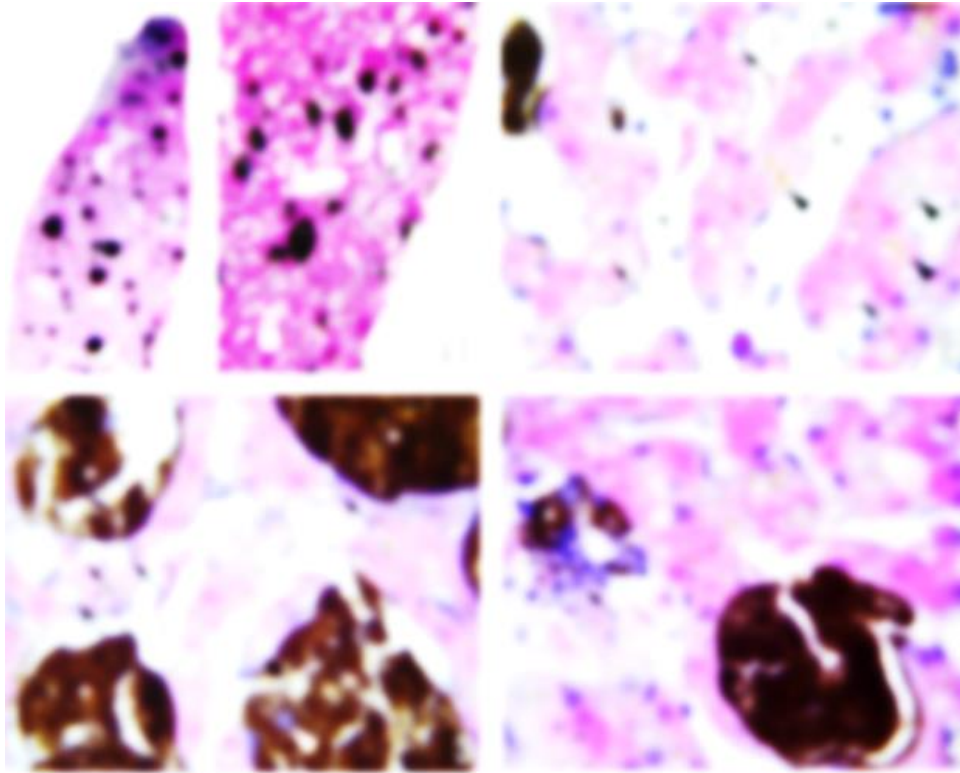
Figure 21. Photomicrographs of the liver of *Scincus scincus*

1. The liver tissue is scaterred by different sized dark spots, bile ducts (asterisks)
2. Cords of hepatocyts alterned by sinusoids. Hepatocytes cytoplasm is vaccuolated.
Note several light vaccuols (arrows) and some ones contain chromogen materiel (arrowheads).
3. Dark spots (asterisks) appear brown are important in size and represent melanomacrophages
centers, containing nulei (arrowheads), near these spots hepatocytes present vaccuoles sticked
to their nuclei (arrows).
4. A cluster of seemingly imflamatory cells (arrow) is observed near the chromogen materiel
(asterisk).
Hematoxylin & Eosin staining.
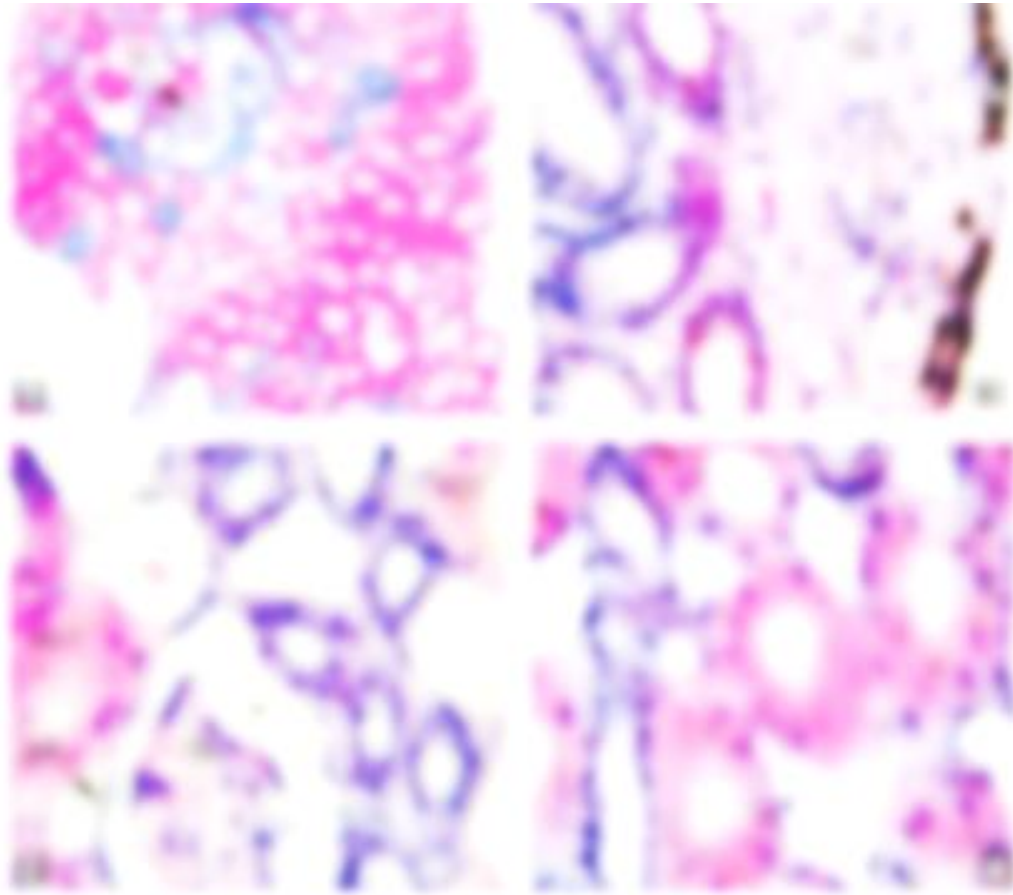
Figure 22. Photomicrographs demonstrating kidney parenchyma microanatomy

1, 2. Zone demonstrating different parts of Nephron: Malpeghian corpuscule
composed of glomerulus (GL), parietal layer (pL), Bowman's space (asterisk)
and tubular elements : proximal convoluted tubule (PCT) and distal convoluted tubule
(DCT). 3. The kideny capsule (Cp) shows chromogen materiel at the edge (asterisk).
4. Note the presence of brown materiel inside PC cells.
Hematoxylin & Eosin stainning
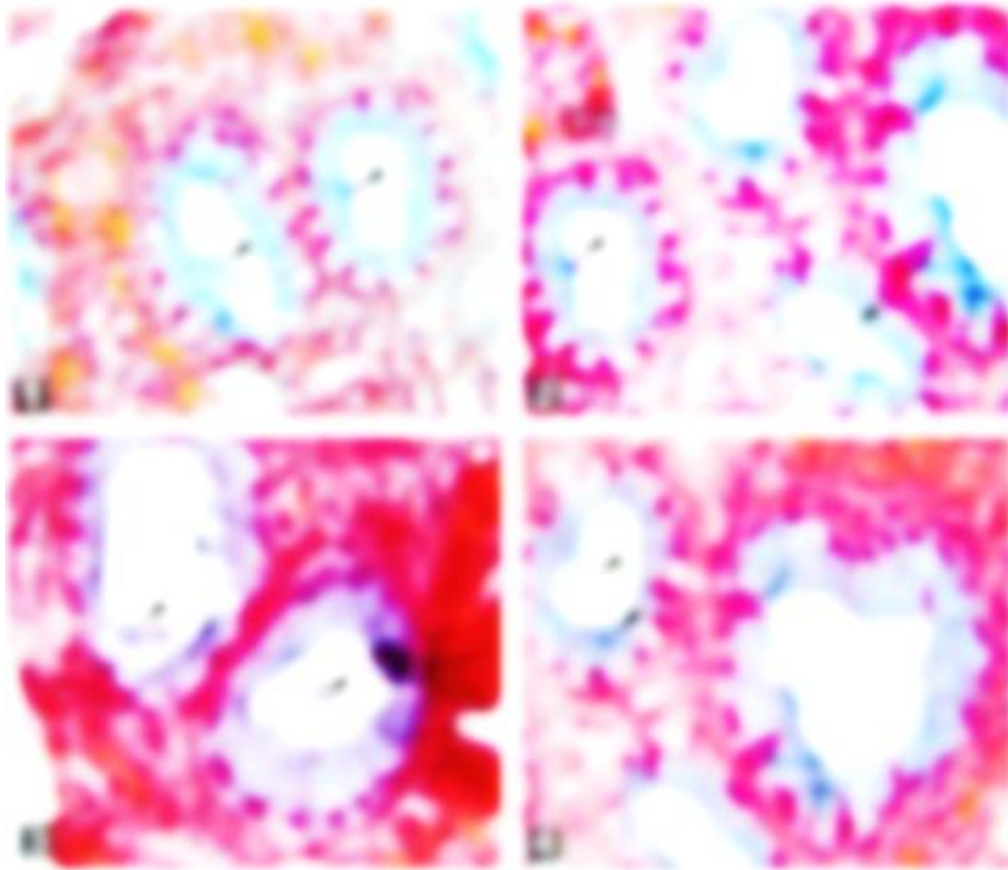
Figure 23. Photomicrographs demonstrating loss of tubular epithelial cells

1, 2, 3, 4. Epithelial tubular loss is observed in nephron's tubules (arrowheads),
note the presence of an important lumen area, a yelow colored materiel in collecting ducts
(CD) (in 1)and a important interstitiel space (asterisks) (in 4 and 5).
Masson' s trichrom staining.
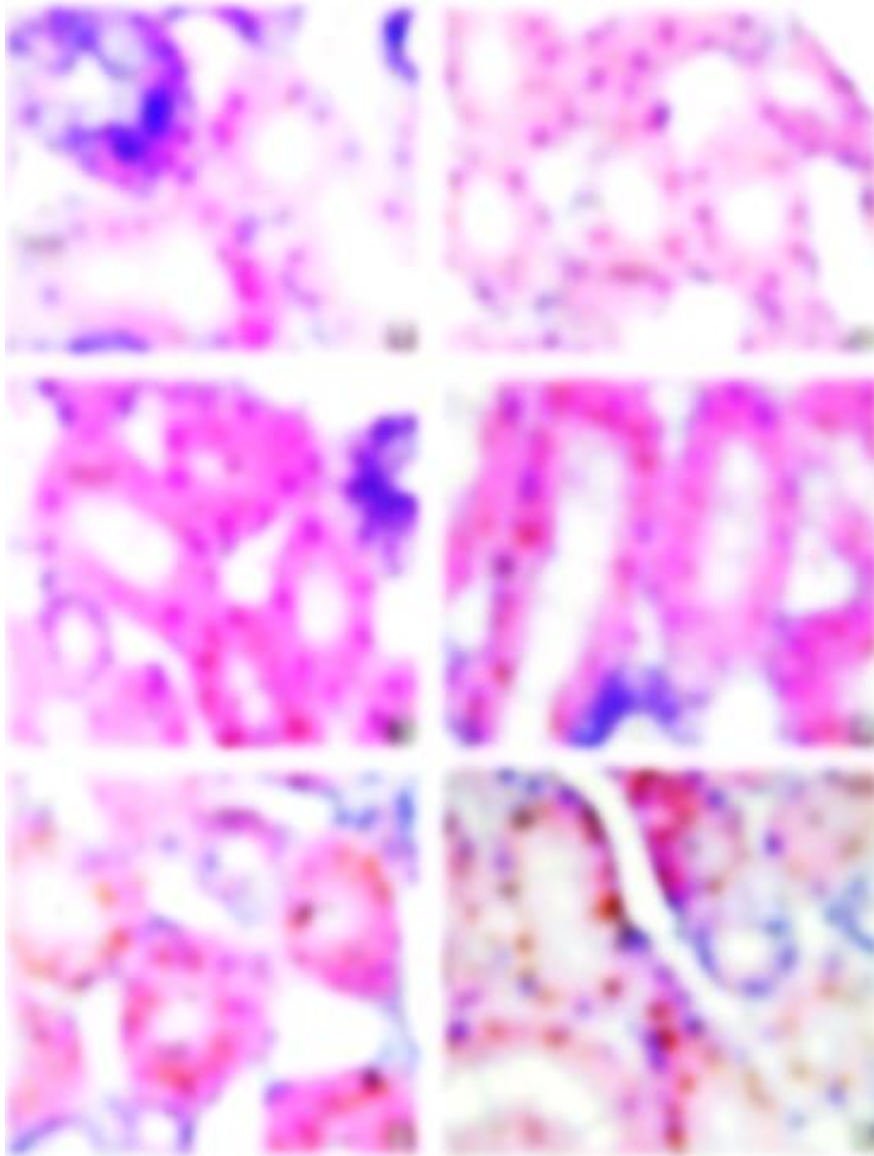
Figure 24. Photomicrographs demonstrationg tissular and cellular abnormalities in kidney

1. Sings of cellular vaccuolisation (asterisk) and debris (circule).
2. Proximal convoluted tubule (PCT) containing picnotic nuclei (condenced chromatine) (rectangle) and debris (circule).
3, 4. PCT presenting degenerating cells (circules)
5. Degenerating cells are observed in collecting ducts (CD) and in convoluted tubule (DCT) (white circule). Asterisks represent obliterated lumen in CD. Note the presence of light vaccuols in CD (arrowheads).
6. CD lumen seems totally erased (asterisks). Note brownish dark materiel essentially in kideny tubules.
Hematoxylin & Eosin staining

II. HISTOPATHOLOGICAL FINDINGS IN *S. SCINCUS* TISSUES

Tissues of interest were analysed using classical histological techniques, namely: Haematoxylin-eosin staining, Masson's trichrome, and Nissl straining.

II.1. Histopathological signs in brain tissue

Figure 19 represents brain tissue stained using Nissl stain. Different abnormalities have been observed in different locations, namely: Irregular plaques were observed far from perikaryons (white matter) Fig. 19.1 and Fig. 19.2, Other irregular material, were found next to a cluster of neurons (Fig. 19.3), plaques of diffused material were detected engulfing a seemingly degenerating neuron, near capillaries (Fig. 19.4). In Fig. 19.5 and Fig. 19.6 abnormal structures are found close to a group of neurons.

II.2. Histopathological signs in skeletal muscle tissue

Fig20.1 and 20.2 represent the general aspect of S. *scincus* skeletal tissue. The skeletal muscle tissue was found to enclose a set of aberrancies, mainly: they presented in Fig. 20.3, uncommon filiform dark structures, the presence of several rifts within muscle cells Fig. 20.4 and Fig. 20.5 (in comparison to other fibers of the same tissue), Some parts of muscle them appear to be separated by spaces from intercalated discs Fig. 20.6. Another noticeable aspect was the irregular plaques taking place at different sites, namely: at the edge of a muscle fiber Fig. 20.6, at the intercalated skeletal muscle fibers Fig. 20.8, and inside muscle cells Fig. 20.9. Numerous satellite cells are shown in the interstitial conjunctive tissue Fig. 20.10, and 20.11. Fig. 20.12 show scattered and numerous nuclei inside the cell fiber.

II.3. Histopathological signs in liver

The liver of *S.scincus* was characterized by remarkable dark spots of melanomacrophages of varying sizes, all over the tissue (Fig. 21.1). Hepatocytes have shown the presence of numerous vacuoles of different sizes, while some of them appear empty, others contain coloured materials (Fig. 21.2). Close to melanomacrophages centers, hepatocytes are observed to contain large light vacuoles close to their nuclei (Fig. 21.3). In certain zones, diffuse brown materials are found with groups of seemingly inflammatory cells (Fig. 21.4).

II.4. Histopathological signs in kidneys

Figure 22 demonstrates the general aspect of *S.scincus* kidneys. The proximal convoluted tubule (PCT) has prismatic epithelial cells that are eosinophil and attract eosin, contrastingly, the distal convoluted tubule (DCT) is basophil and attracts haematoxylin (Fig. 22.1 and Fig. 22.2). The Malpighi corpuscle is characterized by a marked bowman's space (Fig. 22.2). Epithelial cells of PCT contain a brownish material in their cytoplasm (Fig. 22.4).

Figure 23.1 have shown the presence of yellowish material in collecting ducts of *S.scincus* kidneys. Another observable abnormality consists of tubular epithelial cellular loss, and a lumen enlargement (Fig. 23.2 and Fig. 23.3). Additionally, a distinguishable feature is represented by an important conjunctive interstitial space between tubules (Fig. 23.4).

At a higher magnification, different aberrancies were observed in epithelial cells of kidneys' tubules (Fig. 24): Vacuolated cytoplasm and debris (Fig. 24.1), some tubule cells present pycnotic nuclei, along some debris (Fig. 24.2), degenerating cells in PCT (Fig. 24.3, and Fig. 24.4), vacuolated cytoplasm, and degenerating cells in collecting duct (CD), along numerous brown spots (Fig. 24.5 and Fig. 24.6).

CHAPTER IV

DISCUSSION

1. Heavy metals charge in *S. scincus* tissues

Quantification results of heavy metals in the different types of tissues of *S.scincus* have revealed that the kidneys and liver contain especially excessive quantities of mercury, selenium, aluminium, and nickel. Contrastingly, the skin and muscles are distinguished with very high amounts of arsenic, lead, and cadmium. The same results denote the presence of cobalt, copper, and zinc similarly in both types of tissues (liver and kidneys & skin and muscles). The high rates of heavy metals found in tissues of *S.scincus* suggest a considerable bioaccumulation of these elements, through the lizard's eating behaviour (insectivory with contaminated soil uptake) from one side, and due to its permanent contact with contaminated sand, from another side.

Our hypothesis is based on the presence of the studied area in one of the large physical sets of the Algerian sahara (eastern grand erg), comprising the oil producing zone of Hassi Messaoud. The latter, represents one of the largest industrial zones of petroleum in Africa, and consequently one of the intensely attained regions by soil pollution of industrial origins. Furthermore, we suppose that the eolian swirling dominating the eastern grand erg is the origin of transport and deposit of contaminated soil particles, as indicated by Mainguet and Chemin (1984) (Fig. 25).

Les grands ensembles physiques du Sahara algérien (Kouzmine,2007)
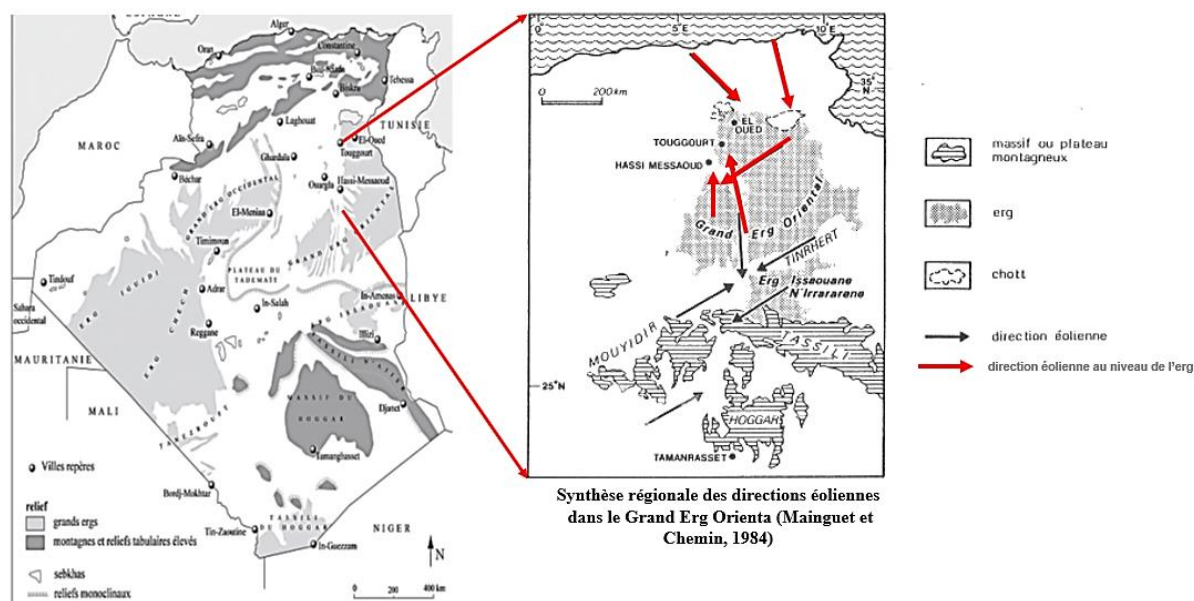
Figure 25. Hypothesis of heavy metal swirl in the eastern grand erg: pollutants sources

The advanced hypothesis joins the conclusion of Benhaddya (2014), who has collected and analysed soil surface samples of 58 sites from different functional zones at Hassi Messaoud, in order to evaluate contamination levels by heavy metals. Results have shown that the average concentrations of Cu, Ni, Mn, Pb, and Zn in examined soils were 13.17, 35.78, 121.21, 130.97, and 61.08 mg/kg respectively. While Ni

concentrations were comparable to ground values, the concentrations of Cu, Mn, Pb, and Zn were higher than reference values. Following the estimation of the integrated pollution index, high concentrations of Pb, and Zn were found in the soils of Hassi Messaoud. Furthermore, these soils are also lightly contaminated by Cu, and Mn.

In another study, Masson et al. (2004), mentioned that the lithometeors, and "Sirocco", or more commonly "rain and red mud", have in common the same eolien transport phenomena associating erosion and deposit of desertic particles. The same authors, have reported on the 21st of February 2004 that half of south France was affected by a meteorological event of eolien transport of saharian dust particles. The records of atmospheric dustiness and resulting dust deposits, revealed an episode of exceptional magnitude. In a few hours, deposits' thickness exceeded 1mm (up to 4 mm in Corse) with a maximum surface charge of 50 g/m^2 (being 50 tons per km^2). In final, 2 million of tons were deposited on a portion of territory located at the south of a line going from Nantes to Besançon.

2. Histopathological signs of heavy metals accumulation *Scincus*

Following the histological analysis of heavy metal contaminated tissues of studied sandfish, diverse cellular and tissular aspects were observed. Results have revealed several abnormalities in tissular, cellular, and sub-cellular compartments.

When ingested into the body, heavy metals are acidified by the stomach's acids. They are oxidized into their oxidative states (Zn^{2+}, Cd^{2+}, Pb^{2+}, As^{2+}, As^{3+}, Hg^{2+}, etc.), these states can easily bind to biomolecules of the body, such as enzymes and proteins by forming stable and strong bounds. Heavy metals most commonly bind to thio groups, the SH group of cysteine, and SCH$_3$ group of methionine. Thus, they can exert their toxicity through numerous mechanisms (Azeh Engwa et al., 2019):

(i) oxidized heavy metals can replace the hydrogen of SH group, and the methyl of SCH3 group, leading to the inhibition of the protein/enzyme activity (Fig. 26).
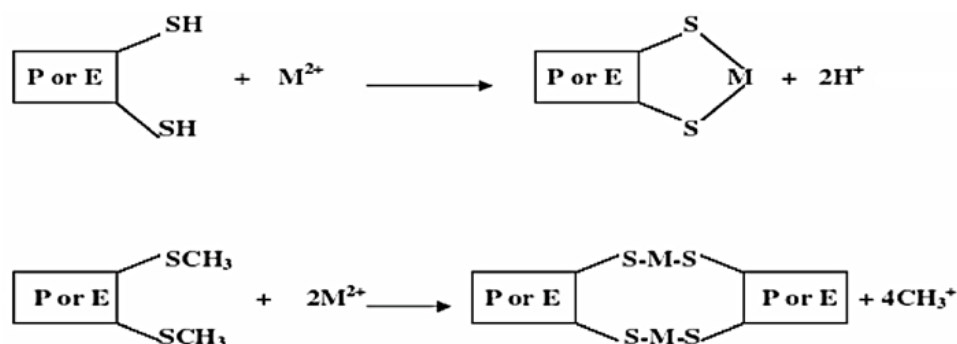

Figure 26. Possible interactions between oxidized metals (M) and proteins (P) or enzymes (E) (Azeh Engwa et al., 2019)

(ii) Heavy metals in their ionic form could substitute in a metalloenzyme other metal ion of a similar size. Similar substitutions can transform enzymes structurally

into an inactive form, thus, altering their activity. Metallic ions, such as: Pb^{2+}, Cd^{2+}, Hg^{2+} and As^{3+} make stable biotoxic complexes with enzymes and proteins, which would be difficult to dissociate.

(iii) They can cause protein aggregations. Yeast cells were observed to aggregate after their exposure to toxic concentrations of arsenite, chromium, and cadmium. Thus, their capacity to trigger and induce protein aggregation most probably relies on their biological actions, as well as their uptake/export from cells.

In kidneys, the marked bowman space that was observed, may reveal an alteration at the glomeruli level that may be due to cell loss, exhibiting similarities with the Fanconi syndrome. It has been reported that most heavy metals, such as Cd^{2+}, Hg^{2+}, and Pb^{2+} compete with zinc and iron on reabsorption metal transporters (MT/ZnT1) (Fig. 27), induce nephropathy with a Fanconi syndrome aspect, and proximal tubular necrosis with a decrease in glomerular filtration rate (Barbier et al., 2005) which were observed in the kidney samples.
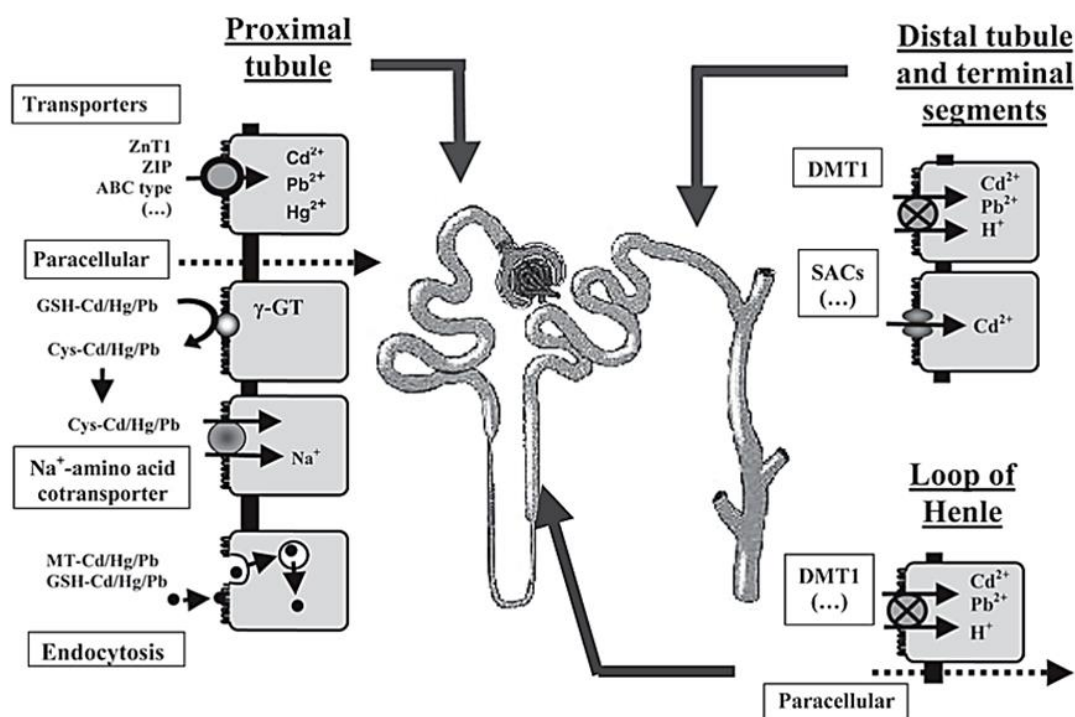
Figure 27. Molecular basis of heavy metals excretion/reabsorption in nephrons
(Barbier et al., 2005)

Another histopathological aspect was represented by a tubular epithelial cellular loss with a luminal enlargement, which could also be explained by the effect of heavy metals reported by (Barbier et al., 2005), who stated that low levels of mercury and lead, over a prolonged period can cause glomerular and tubular damage, and a rapid obstruction of the tubular system, which was also observed at the level of collecting ducts.

A different sign of cell damage was observed in the tissue, represented as pycnotic nuclei and altered cells. This manifestation could be due to Lead, mercury, cadmium, and arsenic toxic effects on the DNA and cellular integrity. Evidence based studies have shown that lead-generated ROS are crucial in chromosomal structure disruption and sequence alteration, Arsenic on the other hand, has been reported to induce histone damage. Mercury has also been reported to cause DNA structural alterations, while cadmium has been described to promote apoptosis. (Azeh Engwa et al., 2019).

The coloured spots in tubular cells may be hyalin droplets. According to (Sato et al., 2005), hyalin droplets can be a renal tubular necrosis marker, and indicate functional disorder of protein reabsorption caused by the degenerating proximal tubular epithelium. These tubular alterations are, indeed, observed throughout the tissue's tubules.

As regards the liver tissue, melanomacrophage centers (MMCs), which are aggregates of substantially pigmented phagocytes, are suggested to be structurally, cellularly, and molecularly similar to mammalian germinal centers, and thus, hypothetically participate in the humoral adaptive immune response. Their cells have various functions, such as melanin synthesis, phagocytosis, free radicals neutralization, and detoxification. MMCs have been shown to respond to the animal's body condition, and pollution exposure (Steinel and Bolnick, 2017). Another study, has suggested that MMCs may increase in number and size under conditions of stress (Ribeiro et al., 2011).

Through the SAA analysis, together with the histological study, it has been confirmed that the lizards were exposed to environmental pollution, and heavy metal toxicity affections in their tissues. We suppose that the abundance and large size of melanomacrophage centers, may be due to the oxidative stress heavy metals induce, through the mechanisms described by (Jomova and Valko, 2011), which might have over-solicited their detoxification, and free radicals neutralization actions leading to their abundance.

The yellow spots observed throughout the tissue may be signs of cholestasis, which is the result of hepatocellular dysfunction or biliary obstruction. The etiology of this dysfunction could be the hepatocellular damage induced by xenobiotics (Raul S. Gonzalez, 2021). As regards the white circles, they may be lipid reserves, which might be a sign of hepatic lipidosis. The latter, is characterized by excessive lipid collection in hepatocytes. This condition is commonly diagnosed in reptiles, and is described as a metabolic condition, instead of a true disease. Because, different species have different degrees of tolerance to lipidosis (M. Silvestre, 2013). We suppose that this was due to the "*ad labium*" food access to these animals, and the lack of activity due to them being in captivity for three weeks.

The Brain tissue have shown several plaques of varying aspects. As regards the round structures, they share similar structural features with Alzheimer type II astrocytes. If these structures were Alzheimer type II astrocytes, then the brain might have gone through a hepatic encephalopathy episode. Certainly that, certain neurons appear to be "anoxic" with pycnotic nuclei (shrunken) (C. Klatt, 2005). However, mercury toxicosis has been reported to induce similar changes, as reported by (Gwaltney-Brant, 2013).

Red spots have been observed around a neuron, it is suggested that they consist of protein aggregates, that have accumulated through the mechanism illustrated in fig.28. It is important to note that metals may also have the ability to hinder protein folding. Mercury, cadmium and lead are correlated with efficiency in inhibiting protein folding in vitro, their actions are due to them forming stable complexes with imidazole, thiol and carboxylate groups in proteins. The aggregates could also be due to the formation of biotoxic complexes, that are formed by heavy metal ions strongly binding to proteins and creating difficult to dissociate complexes in tissues (Azeh Engwa et al., 2019).
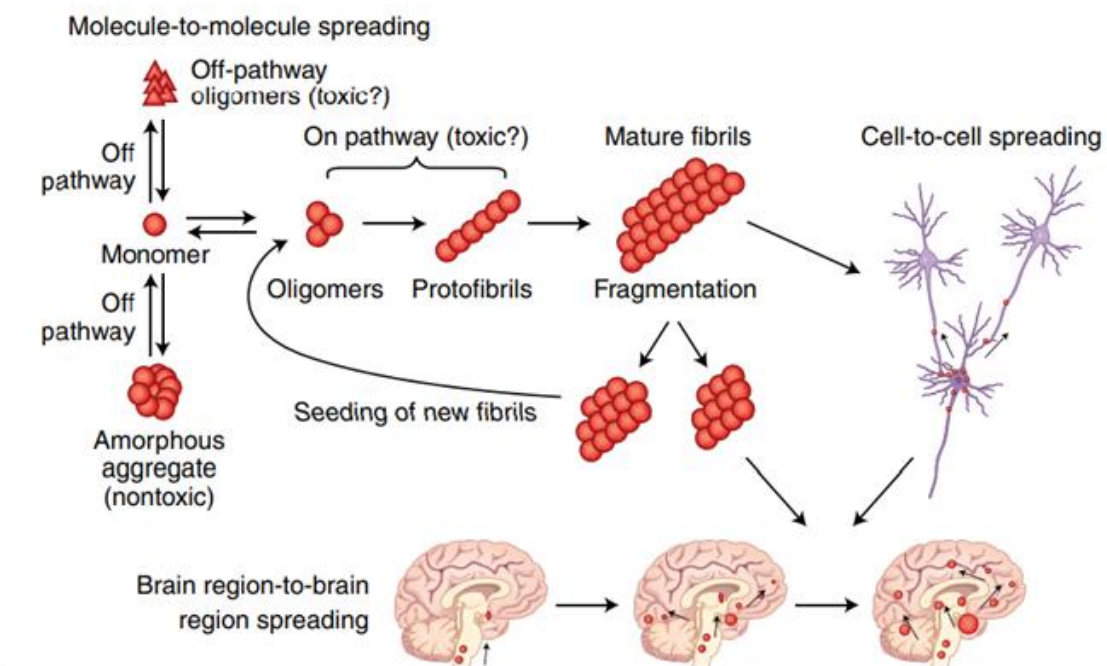
Figure 28. protein aggregation mechanism (Soto and Pritzkow, 2018)

In the skeletal muscles, a group of cells was observed in the interstitial space of muscle parenchyma, it is suggested that these participate in an inflammatory reaction, or in tissue maintenance. Certainly that, there is an abundance of satellite cells in the tissue, which we suppose is due to the different observed alterations that may require restauration and maintenance by satellite cells as clarified by (Dumont et al., 2015). In comparison to (Canei and Nonclercq, 2021) findings on the skeletal muscle structure

of a healthy S.scincus animal, our study lizard exhibited numerous structural alterations, represented by numerous rifts,

As regards the filiform dark structures, it is suggested that they may be protein aggregates due to heavy metals' effect, or alterations that are due to heavy metals interfering with the usual functioning of enzymes/proteins and enzymes (Fig. 29), through the following mechanism: when the protein is bond to a heavy metal and forms the specific complex with its specific enzyme, the product is not formed, and the complex (protein-enzyme) is not dissociated, thus, the enzyme becomes blocked, keeping the heavy metal in the tissue leading to dysfunction, and aberrancies in the body. The inhibition of a protein such as thiol transferase can yield an increase in oxidative stress and cellular damage. For instance, arsenic incoming from fungicides, herbicides and insecticides can bond with –SH enzyme groups, and inhibit their catalytic activities (Azeh Engwa et al., 2019). A previous study has analysed the potential effect of pesticides on the enzymatic function of S.scincus tissues, in El Oued region (Kechida et al., 2020), it has concluded that the catalase enzyme had a significative elevated activity. Catalase enzyme activity had been described to be strongly influenced by trace metal elements concentration in tissues (Wołonciej et al., 2016).
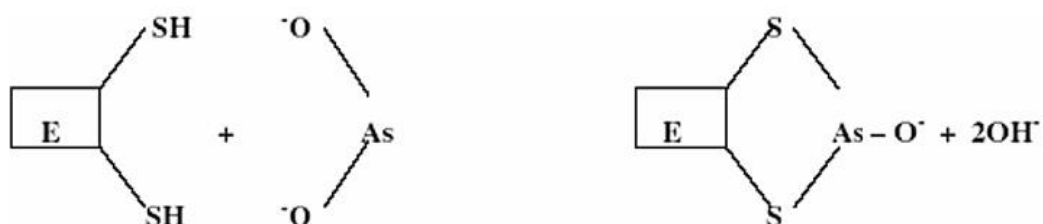

Figure 29. Interaction of arsenic with a thio group (Azeh Engwa et al., 2019)

CONCLUSION
AND
PERSPECTIVES

Conclusion

In the present study, it has been demonstrated that *S.scincus* lizards share the heavy metal bioaccumulative property that was described in other lizards. Thus, *S.scincus* species may, indeed, provide a good animal model for the assessment of environmental pollution in their natural habitat. This analysis has also revealed that El Oued Souf area is most likely polluted with alarming rates of heavy metals, which is likely to result from anthropogenic activities, bad waste management, as well as wind movement and soil particle deposits between other contaminated areas, such as Hassi Messaoud and this region.

Results have shown that the consumption of more than one lizard in a day could provide a high charge of toxic heavy metals to the consumer's organism, exceeding WHO maximal daily recommendations. Consequently, the recurrent administration of this animal's flesh into the body could induce a chronic toxicity of Arsenic, lead, and other heavy elements. Applied topical creams that are based on the *S.scincus* dried powder could equally present a risk of metal toxicity, since many heavy metals are known to reach the bloodstream through dermal absorption, such as arsenic. Infants are more vulnerable to heavy metals toxicity than adults, thus, the sandfish consumption in any form is highly discouraged, certainly that many heavy metals have been shown to interfere with neurological development of children, such as lead, mercury, and arsenic.

Histological results have shown the extent of damage skinks are suffering from, mostly due to their polluted environment. The kidney function was the most adversely affected, which suggests that the damage was most likely induced by toxicants that are meant to be excreted. There were also signs of biliary dysfunction, which is also responsible for the excretion of certain toxicants. It has been established that heavy metals, like copper, and cadmium have a strong capacity of generating oxidative stress, creating biotoxic complexes with proteins, hindering enzymatic functions, substituting other co-factors in essential metabolic reactions and inhibiting them, damaging the DNA and altering its repair mechanisms, histone damage, in addition to neurodegenerative processes, and disruptions.

The most concerning danger of consuming sandfish is chronic exposure to a variety of heavy metals in high enough doses, together at once. As a result, the consumption of *S.scincus* is strongly discouraged due to the dangerous heavy metals exposure risk. Certainly, that humans are higher in the food chain, and could biomagnify these metals by consuming lizards that are themselves magnifying the metals of their environment.

CONCLUSION

Further work may be carried on the histopathological aspects observed in *S.scincus* lizards' tissues, mainly an electron microscopy study, paired with specific oxidative stress biomarkers, in order to identify more specific causes to the observed magnified adverse effects. It is also recommended to pair clinical data related to kidney disease of idiopathic nature with heavy metal toxicity.

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ANNEX

ANNEX

1. Preparation of gelatin-coated slides

The gelatin-coating solution was prepared by dissolving 2.50g of gelatin in 500ml of heated distilled water (without exceeding 45°C). After the gelatin had dissolved, 0.25g of chromium potassium sulfate was added to the solution. The solution was then filtered using filtering paper.

First, slides were put in alcohol 96% overnight. The following day, they were cleaned in soapy water then washed, first in tap water and finally in distilled water. After that, slides were put in metal racks and dipped in the gelatin-coating solution.

Further, the dipped metal racks are drained of excess coating solution. After that, the slides were transferred into a slide box, then covered (to stay protected from dust) and left to try at room temperature for 48 hours.

2. Phosphate buffered saline preparation

10 times Phosphate buffer saline was made by combining: 80g of sodium chloride (NaCl), 2g of potassium chloride (KCl), 18.1g of sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), 2.4g of of potassium dihydrogen phosphate (KH_2PO_4) and 800mL of distilled water. Subsequently the pH was adjusted to 7.4 with hydrogen chloride solution of 2N (HCL). Then, distilled water was used to complete the volume to 1L. The solution was autoclaved for 20 minutes and stored.

3. Paraformaldehyde solution preparation (PAF)

To prepare the PAF solution, 16g of paraformaldehyde powder was put in 190ml of distilled water. The solution then was stirred using a magnetic heat/stir plate at 65°C. at clearance, the pH was adjusted to 7.2 using a 2N NaOH solution.

4. Nissl staining solution preparation

Solution A: was prepared by dissolving 13.6g of granular sodium acetate in 92mL of distilled water.

Solution B: was prepared by mixing 29mL of glacial acetic acid with 471mL of water.

The staining solution was prepared by combining: 2.5g of cresyl violet, 300mL of distilled water, 30mL of 1.0M sodium acetate (solution A), and 170mL of 1.0M acetic acid (solution B). The solution is stirred for 7 days, and then filtered.

4.1. Staining procedure

Deparaffinize and hydrate to distilled water.

2. Cresyl violet, two minutes.

3. Wash in distilled water.

4. Dehydrate, clear in xylene, coverslip.

5. Carazzi's Hematoxylin

This is an alum hematoxylin which is chemically ripened using potassium iodate. The staining solution was prepared by combining 5g hematoxylin with 100 ml glycerol, and then with 25g potassium alum dissolved in 400 ml distilled water and 0.1 g potassium iodate. Let repent for 1 to 3 days and then filter. The coupes are immersed for 10 min.

6. Masson's Trichrom

- Solution A: modified hematoxylin: comibinaition of Chromium alum 0, 1g, 5 mg hematoxylin , 5 ml Sulfuric acid 10%, 0, 55 calcium carbonate   and 250 ml Distilled water .

Heat the distilled water, add the chromium alum and boil until a green color appears, After cooling, add the hematoxylin mixed to 10% sulfuric acid, afterward, add calcium carbonate to the mixture, leave to cool, then filter.

- Solution B: Fuchsine-Ponceau: Comibinaition of 100 ml Acid fuchsin and 0.5 ml acetic acid and 1g red Ponceau and 100 ml disstiled water.

 Prepare the two solutions Fuchsine and Ponceau: Mix one part of the Fuchsin/acetic acid solution with two parts of the red Ponceau/ distilled water.

- Solution C: add 3g of phosphomolybdic acid and 2g of orange G to 100 ml of Distilled water. Mix to obtain an orange solution than leave to stand for an hour and filter.

- Aniline Blue stain: mix 3g aniline blue in 2.5 ml of Acetic acid and 100 ml of distilled water then Leave to stand for an hour and filter.

6.1. Procedure of staining

 Deparaffinize and hydrate.

• Stain with modified hematoxylin 02min

• Wash with distilled water

• Color with the fuchsin ponceau mixture.02min

• Rinse with 1% acetified water

• Colored with orange G phosphomolybdic acid 02min

• Rinse with 1% acetified water

• Color with aniline 15sec to 30 sec

• Rinse with 1% acetified water

• Dehydrate and coverslip