Emerging Contaminants and Associated Treatment Technologies

Biljana Balabanova
Trajce Stafilov *Editors*

Contaminant Levels and Ecological Effects

Understanding and Predicting with Chemometric Methods

Springer

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Preface

The biosphere is the medium that is naturally optimized for the growth and development of a huge number of biological organisms. Nature itself creates natural disasters that degrade the ecosystems and organisms that live in it. Humans, in spite of their existential question in nature, still continuously degrade nature and its living environment. This anthropogenic factor affects all segments of the environment, the lower parts of the atmosphere, the upper parts of the lithosphere, as well as the hydrosphere. Intensive technological development as well as the availability of natural raw materials for their utilization have significantly enabled the progress of these degradation processes in nature. On the other hand, the chemical and pharmaceutical industry, with their intensive development, have generated substances that are not only unnatural but also highly risky for the human population and the environment. Pollutants and potentially toxic substances are continuously introduced into all segments of the biosphere, shifting the natural balance of natural normal distributions. The environmental pollutants create abnormal media for living organisms. These days, we are increasingly faced with the fact of the ecological risk for the survival of many species. In recent decades, researchers have paid great attention to environmental risk, determining the pollution index and identifying polluted sites where it is necessary to prevent further degradation. Therefore, several critical aspects should be involved when we initiate and launch environmental research or monitoring. One of the aims of the modern environmental investigations is to obtain more objective data for the complex but silent environmental markers, which will be identified as typical pollutants in various parts of the environment. The modern analytical approaches involve sophisticated and sensitive instrumental technique, but the main question is how to create a corresponding data matrix and proper data analysis. Chemometrics is a routine chemical sub-discipline, which involves several mathematical methods for extracting more realistic and proper environmental information. The implementation of modern and novel chemometric methods becomes a critical point in the environmental studies these days. Therefore, this book summarizes the latest investigations of the concerning parts of the biosphere, affected with hazards substances. Moreover, selected case studies investigation with spacious applicability will create general framework of the opportunities, advantages, weaknesses, and anomalies of the mathematical approaches of the analysts. Furthermore, a properly defined chemometric model of each environmental investigation will provide long-term applicability potential.

This book consists of 13 chapters contributed by relevant experts in various fields correlated with environmental issues. All the chapters are logically selected and arranged to provide comprehensive state-of-the-art information about the practical aspects of environmental chemometric approaches. In this volume, the introductory chapter gives an overview of the critical environmental issues, such as degradation, ecological risks, and silent hazards. The next five chapters are on air pollution aspects: pollutants, hazardous emissions, monitoring, indication, as well as spatial indication of emission sources. This chapters give attention to air pollution, air deposition, and distribution models. Certain emphasis is given to the moss effectiveness for bioindication of the potential ecological risk. Another chapter is dedicated to the application of lichens as the main indicator in biological monitoring of air quality. Water pollutants and their determination issues are the main topics in the next two chapters, covering key issues in spatial distribution of various metals in different parts of the environment. These chapters deals with improving effective analytical methodologies of GC–MS and ICP-MS for tracking potential contaminants. This is followed by research into the effective removal of toxic hazard from aquatic systems. The next group of chapters is dedicated to the state of chemical characterization of the plant food and endemic plant species as characteristic media that involves the potential ecological risks. The presented are multidisciplinary approaches which enable detailed and precise elaboration of the set research subject.

All the chapters and their contents are supported by extensive citation of available literature; calculation and assumptions are based on realistic facts and figures of the present status of research and development in this field. This book will provide a wealth of information based on a realistic evaluation of contemporary development in environmental investigations with special emphasis on the latest research studies. Furthermore, this book also highlights the potential and perspective use of the multidisciplinary aspect for enchasing environmental pollution and potential ecological risks.

Most of the chapters cover advanced research as well as the use of more sophisticated methodologies. Therefore, we believe that the usefulness of this book will be primarily directed to experienced researchers. But of course we also encourage young researchers to use the book, because in many of the chapters, the methodologies used by the authors are explained very basically. We also believe that certain institutions and state regulatory bodies can use this book as an initiator for critical issues related to environmental degradation, environmental risks and their determination, and future prevention.

Štip, Republic of North Macedonia Biljana Balabanova
Skopje, Republic of North Macedonia Trajče Stafilov

List of Abbreviations

AAS	Atomic absorption spectrometry
AED	Atomic emission detector
AES	Atomic emission spectrometry
AF	Attenuation factor
AFR	Revised attenuation factor
AFT	Log-transformed attenuation factor
ALA	Alpha linolenicacid
ANN	Artificial neural networks
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ATP	Adenosine triphosphate
BAF	Biological accumulation factor
BTF	Biotransfer factor
CA	Cluster analysis
CART	Classification and regression trees
CDI	Chronic daily intake dose
CEC	Cation exchange capacity
CHCA	A-cyano-4-hydroxycinnamic acid
CR	Carcinogenic risk
CVAAS	Cold vapor atomic absorption spectrometry
DBCP	Dibromochloropropane
DCM	Dichloromethane
DHA	Docosahexaenoic
DMT	Digital terrain models
DRC	Dynamic reaction cell
DTPA	Diethylenetriamine pentaacetic acid
DW	Dry weight
EC	Elemental carbon
ECD	Electron capture
EDB	Ethylene dibromide
EDS	Energy dispersive X-ray spectrometry

EEA	European Environment Agency
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EPA	Eicosapentaenoic
ESI	Electrospray ionization
ETAAS	Electrothermal atomic absorption spectrometry
FID	Flame ionization detector
FS	Fluorescence spectroscopy
GC	Gas chromatography
GHG	Greenhouse gas
GLI	Global leachability index
GUS	Groundwater ubiquity score
GWCP	Groundwater contamination potential
HI	Hornsby index
HPLC	High-performance liquid chromatography
ICP–AES	Inductively coupled plasma – atomic emission spectrometry
ICP–MS	Inductively coupled plasma – mass spectrometry
IR	Infrared spectroscopy
IRMS	Isotope ratio spectrometry
LA	Linoleic acid
LC	Liquid chromatography
LDA	Linear discriminant analysis
LEACH	Leaching index
LIX	Screening leachability index
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPI	Leaching potential index
LSD	Least significant differences
MAC	Maximum permissible concentrations
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MSA	Multivariate statistical analysis
NMR	Nuclear magnetic resonance
ORS	Octopole reaction system
PC	Principal components
PCA	Principal component analysis
PDA	Photodiode array
PDO	Protected designation of origin
PGI	Protected geographical indication
PLP	Pesticide leaching potential index
PTFE	Polytetrafluoroethylene
PTH	Parathyroid hormone
PTR-MS	Proton transfer reaction mass spectrometry
RAF	Relative accumulation factors

REEs	Rare earth elements
RLP	Relative leaching potential index
SA	Sinapic acid
SEM	Scanning electron microscopy
TDS	Total dissolved solids
TEP	Thermoelectric power plant
TF	Translocation factor
TGA/DTA	Thermogravimetric and differential thermal analysis
TIN	Triangular irregular network
TLC	Thin layer chromatography
TOF	Time-of-flight
TPP	Triphenylphosphate
VI	Volatility index

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Chapter 1
General Aspects of Environmental Degradation vs. Technological Development Progression

Biljana Balabanova

Abstract A look at natural resources in terms of life cycle reveals several environmental problems related to production and consumption and combines resource use and waste generation. The use of resources and the generation of waste are special impacts on the environment, but the two issues share many of the same driving forces – largely related to how and where we produce and consume goods and how we use natural capital for economic sustainability development and consumption. More recently, life cycle thinking has been introduced as the guiding principle of resource management. Environmental impacts are considered throughout the life cycle of products and services to avoid or minimize the transfer of environmental stress between different stages of the life cycle and from one country to another. With the passage of time and the long-term activities of the human factor, the contents of certain metals have been completely and permanently changed in relation to their natural existence in the environment. These changes can have a significant influence on the physiology and ecology of the organisms adapted to survive in thus created conditions of higher metal contents. The anthropogenic activities for exploitation of natural resources and their processing through adequate technological processes and management of the waste produced by the same represent a global problem of pollution of the environment. The distribution of the different chemical elements, including the potentially toxic metals, creates characteristic conditions for the living organisms. Considering that their contents in the environment are variable, it is important to identify the regions with changed contents, differing from the natural distribution of the elements in the different segments of the biosphere.

Keywords Bio-toxic hazards · Environmental pollution · Mass spectroscopy · Toxicity

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1.1 General Aspects of Environmental Pollution

Environment is the space with all living organisms and natural resources, of the natural and created values, their mutual relations, and the total space in which man lives and in which the settlements, the goods in general use, the industrial, and other objects are located. Environmental pollution is most often the result of introduction of hazards into the air, water or soil, which can reduce the quality of the environment, life and human health; pollution, on the other hand, is a harmful change in the environment that disrupts or affects the biodiversity and survival of flora and fauna, including the human population. The anthropogenic factor enables processes and changes that degrade the soil, which reduces soil fertility. Worldwide, it is done in many ways: by destruction (destruction), which reduces the land fund (erosion, open-pit mining, and excavation of various materials, covering with various waste, conversion into use to build various facilities), then by contamination from other components of the environment (air and water), and eventually by degradation in many ways in agriculture and forestry (Daughton 2005; Brack and Stevens 2001; Blais et al. 2015). Fertile soil is reduced by various types of destruction, such as erosion, open-pit mining and excavation of various materials; then by covering with municipal waste and various wastes from mines, thermal power plants and metallurgical and other industrial facilities; and, finally, a conversion into land use (conversion) with the construction of water reservoirs, settlements and industrial and infrastructure facilities (Uth 1999; Hou and Zhang 2009; Xue and Zeng 2011). In basic, three segments are generally affected in the biosphere: air, soil, and water ecosystems.

1.1.1 Pollution Hotspots in Environment

Air Pollutants released into the air can come from: a) natural sources such as volcanic eruptions, forest fires and chemical reactions, biological sources; b) anthropogenic sources, such as traffic, fossil fuel combustion, discharge of pollutants, greenhouse gases, and other substances that directly or indirectly affect air quality. That is why it is of particular interest to have data on origin, presence, and impact of pollutants present in the air in order to take measures to reduce them. To improve air quality, it is necessary to prepare plans and programs for undertaking protection measures and air quality and emissions management.

The collection and processing of data on air emissions needs to be done continuously throughout the year. Through the data obtained from the emission measurements of the larger companies that emit pollutants into the air, the quantities of pollutants for each country are monitored on an annual basis. The data provide a basis for the preparation of reports and strategies for reducing air emissions, as well as protection and improvement of ambient air quality.

This process is especially important for countries in transition, where processes in industry and urbanization have a growing impact on environmental degradation. Monitoring of emissions of pollutants into the air needs to be in accordance with the legal acts in the country as well as in accordance with international agreements and conventions.

The biggest contributors to the release of basic pollutants (SO_2, NOx, CO, and TSP) are the following sectors:

- Combustion plants for fuel transformation at electricity generation.
- Combustion of fuels for non-industrial purposes-production of heat-heating stations.
- Combustion processes in industry for the purpose of producing heat to run the process.
- Production processes: this sector includes emissions from various production processes, such as the oil industry, production of steel, iron, non-ferrous metals, cement, etc.
- Production and distribution of fossil fuels and geothermal energy.
- Use of solvents and other products.
- Passenger traffic in which the emissions during the combustion of fuels in the means of transport are given.
- Emissions from fuel combustion in railways, air traffic, agricultural machinery, etc.
- Emissions from waste and waste disposal, incineration of waste in open landfills, and acceleration or other processing of waste.
- Agriculture, emissions from fertilizer application, fermentation, and use of pesticides.
- Emission from other sources, which may include emissions that are not a consequence of human life, CO_2 emissions, etc.

To monitor the air condition, it is necessary to perform monitoring of pollutants and the same to identify qualitatively and quantitatively. Monitoring has an essential task within the environmental management. Namely, it is a basis for undertaking measures for protection against pollution and a means used to improve air quality in the environment. Sulfur dioxide (SO_2) is formed during the combustion of sulfur-containing fuels, such as coal or oil. Certain mineral ores also contain sulfur, and sulfur dioxide is released during their processing. Significant sources of SO_2 are power plants, the oil refinery, and the metallurgical industry. The sulfur content of the fuels used is high and causes occasional high concentrations of SO_2 in the ambient air in cities and industrial zones. Traffic fuels are also high in sulfur and degrade air quality.

Research studies have shown that more nitrogen oxides are present in the air, but the most important are nitrogen dioxide and nitrogen monoxide. These pollutants are usually the result of natural sources. However, in urban areas, they mostly come from traffic and industry. Nitrogen dioxide (NO_2) is a gas that is formed in the process of combustion at high temperatures. Old and irregularly maintained cars produce the highest concentrations of this pollutant (Andreae and Merlet 2001).

Particles with dimensions up to 10 micrometers (PM10) are long retained in the air and are formed as a result of natural and anthropogenic sources. Natural sources include volcanic eruptions, yellow rains, forest fires, and chemical reactions. The most important anthropogenic sources are the combustion of coal, wood, and oil, industrial processes, traffic, and waste incineration. Particularly toxic chemicals are released through uncontrolled incineration of household waste (i.e., incineration of waste in yards), which is common in cities, especially in rural areas (Sarti et al. 2017; Longoria-Rodríguez et al. 2020).

Carbon monoxide (CO) is a toxic gas that occurs as a result of combustion of fuels from transport vehicles, incomplete combustion of fuels in power plants, incomplete combustion of solid waste, and industrial processes (Vallero 2008).

Tropospheric ozone (O_3) is formed by the reaction of pollutants (nitrogen oxides and volatile organic compounds) under the action of sunlight. Sources of such pollution include emissions from vehicles and industrial processes, vapors from gasoline, and chemical solvents. Even rural areas are prone to rising ozone levels as wind carries ozone and pollutants hundreds of miles from the source. Also, organic compounds released from forest areas affect the formation of ozone. However, its content is influenced by the period of the day (intensity of solar radiation) and the seasons. The highest concentrations of this pollutant are observed in spring and summer, while the lowest concentrations are observed during the winter period (Grasimov 2004).

Soil The soil has a number of ecological functions, which are essentially important for the protection of the environment, but also for the economy and the progress of the society as a whole. The impacts on the soil caused by human activities are constantly increasing and lead to degradation and desertification of the land, which causes serious socioeconomic consequences. The main threats to the healthy condition of soils are erosion, local and diffuse contamination, salinization, soil loosening, etc. (Siegel 2002; Jarup 2003; Kabata-Pendias and Mukherjee 2007). Nowadays, there is an increasing need for the adoption of appropriate regulations, especially for developing countries, which will treat the soil in many respects as an environmental medium. It is necessary to define the maximum allowed concentrations in the soils of heavy metals and then certain substances such as pesticides, polycyclic aromatic hydrocarbons, halogen hydrocarbons, and others (Laden-Berger 2012.)

Water Water pollution involves the introduction of various pollutants into rivers, lakes, and groundwater, but also into the seas and oceans. This occurs during direct or indirect introduction of pollutants into the water in the absence of appropriate purification measures and disposal of harmful substances (Fitzpatrick et al. 2007).

In most cases, freshwater pollution is invisible because the pollutants are dissolved in the water. But there are exceptions: foam detergents, but also petroleum products that float on the surface and uncleaned watercourses (riverbeds, canals). There are several natural pollutants. Aluminum compounds found in the ground reach the freshwater system as a result of chemical reactions. Floods absorb magnesium compounds from meadow soils, which cause great damage to fish populations.

But the amount of natural pollutants is negligible compared to man-made substances. Thousands of unpredictable chemicals are introduced into water basins each year, most of which are new chemical compounds (xenobiotics). Increased concentrations of toxic heavy metals (such as cadmium, mercury, lead, and chromium), pesticides, nitrates and phosphates, petroleum products, and surfactants can be detected in water.

As it is known, up to 12 million tons of oil are imported into the seas and oceans every year. Acid rains also play a role in increasing the concentrations of heavy metals in water. They are able to dissolve minerals in the soil, which leads to an increase in the content of heavy metal ions in the water. Nuclear power plants also interfere with radioactive waste in the water cycle. Discharge of untreated wastewater from households leads to microbiological water pollution (Wania and Mackay 1996).

Specific pollutants that pollute water are various chemicals, pathogens, as well as physical or sensory changes such as fever and discoloration. Although many of the regulated chemicals and substances can be found in natural conditions (calcium, sodium, iron, copper, manganese, etc.), concentration is usually key in determining what is a natural constituent of water and what is a pollutant. High concentrations of naturally occurring substances can have adverse effects on aquatic flora and fauna (Wania and Mackay 1996; Rodriguez-Proteau and Grant 2005).

Substances that consume oxygen can be natural substances, such as plant matter (leaves or grass), but also man-made chemicals. Other natural and anthropogenic substances can cause turbidity, which blocks light and impedes plant growth and clogs the gills of some fish species. Many chemicals are toxic. Pathogens can cause waterborne diseases in both humans and animal hosts. Changes in the physical chemistry of water include acidity (change in pH), electrical conductivity, temperature, and eutrophication. Eutrophication is the increase in the concentration of chemical nutrients in a given ecosystem to the extent that it increases the primary production of the ecosystem (Rodriguez-Proteau and Grant 2005). Depending on the degree of eutrophication, there may be subsequent adverse environmental effects such as anoxia (severe oxygen depletion) and severe reductions in water quality, affecting fish and other animal populations. Organic and inorganic substances can act as pollutants in water ecosystems. Organic pollutants of water are:

- Detergents
- Disinfectant by-products found in chemically disinfected drinking water, such as chloroform
- Waste from food production processes, which may be represented by substances that require oxygen, oils, and fats
- Insecticides and herbicides, various organohalides, and other chemical compounds
- Petroleum hydrocarbons, including fuels (petrol, diesel, propellant, and fuel oil) and lubricants (motor oils), as well as by-products of fuel combustion
- Waste from trees and shrubs
- Volatile organic compounds (VOCs) as industrial solvents
- Polychlorinated biphenyls (PCBs)

- Trichloroethylene
- Perchlorate
- Various chemical compounds found in personal care products and cosmetics

Most common inorganic water pollutants and occurrences are:

- Acidity caused by industrial discharges (especially sulfur dioxide from power plants)
- Ammonia from food processing waste
- Chemical waste as an industrial by-product
- Artificial fertilizers containing nutrients (nitrates and phosphates) found in rainwater that passes through agricultural areas, as well as for commercial and domestic use
- Heavy metals from motor vehicles (which reach the rainwater) and discharges from acid ores

Thermal pollution is an increase or decrease in the temperature of a natural water basin and is caused by human influence. Unlike chemicals, thermal pollution results in changes in the physical properties of water. A common cause of thermal pollution is the use of water as a coolant in power plants or industrial production. Rising water temperatures reduce oxygen levels (which can cause fish death) and affect the composition of the ecosystem, for example, by invading thermophilic species (Barceló 2007).

Industry is one of the largest sources of water pollution considering that more than 50% of wastewater comes from industrial industries that often discharge it into surface water directly or indirectly without prior treatment (Barceló 2007; Dvoršćak et al. 2019).

The amount of municipal wastewater depends on the number of households of the population, the organization of communal services for rainwater drainage through sewerage systems and treatment systems, the disposal of municipal waste in appropriate landfills and proper handling, etc. (Richardson and Kimura 2016). Municipal wastewater is completely biodegradable because it is mostly loaded with organic matter, which is mostly of plant and animal origin (Richardson and Kimura 2016). However, due to the changes in the lifestyle of the inhabitants that have occurred in recent decades, the quality of municipal wastewater is changing, especially due to the presence of synthetic detergents for washing, laundry, and other sanitary needs. Pollution of the city communal waters is increasing from the roads due to the use of salt, sand, etc. substances in the winter months (Richardson and Kimura 2016). These substances reach the rivers or water reservoirs by washing the streets directly or through the sewerage network for atmospheric rains (Richardson and Kimura 2016). Municipal communal waters, especially fecal and waters originating from organic landfills, are also polluted biologically with pathogenic viruses, bacteria, parasites, and other microorganisms (Misra et al. 1994; Brack et al. 2016).

Modern agriculture disturbs the environment in many ways. Hedges are being removed to bring in large machinery, which makes agriculture economically viable. This destroys habitats and disrupts complex food chains and their connections.

Farmers use pesticides to kill pests. This increases food production, but destroys wildlife. In addition, pesticides contaminate food intended for humans. Pesticides can be blown into streams and contaminate drinking water. Fertilizers are used as additional nutrients to accelerate plant growth. They can also be washed from soil in lakes and rivers and contaminate drinking water supplies (Petrie et al. 2015).

Pollution of water with fertilizers can lead to pollution of drinking water pools, the most important of which are lakes. Fertilizer causes increased growth of plants in the water (Richardson and Kimura 2016). Those plants that compete for light will die out. Microorganisms feed on dead plants, increasing their numbers. An increasing number of microorganisms consume more oxygen than water. Fish and other aquatic animals die from lack of oxygen.

1.1.2 Bio-toxic Hazards

Toxic effect unites a set of disorders, physiological, biochemical and structural, which occur in the body under the action of a toxic substance. Toxic substances can be defined as the extent to which a certain substance will be toxic depending on the intensity of the processes that take place in the body at the same time: resorption, detoxification, deposition, and elimination. Toxic substances usually enter the human body through the skin, through the mouth and digestive organs, or through the lungs.

After resorption, they reach the blood and then the liver and organs, which are less sensitive to toxic substances. The liver is the most important organ in which toxic substances are broken down by a process called detoxification. Through this process, the products of metabolism, toxic substances, are less toxic and are excreted from the body.

A part of the resorbed toxins reaches the organs that are less sensitive to them and remain there for a longer time (deposition). Thus, many insecticides are retained in the adipose tissue and strontium and lead in bone tissue. One part of the toxin is excreted from the body (elimination) through the kidneys (the largest part), feces (insoluble compounds such as metals), and the lungs (gaseous toxins).

The effect of toxic substances on the body can be:

(a) Local – action of toxins in the immediate place of contact with the organism – skin, mucous membranes, and eyes.
(b) Systemic – the action of toxins after their resorption in the body is manifested on the organs and systems of organs (digestive organs, lungs, blood, etc.).

Short-term exposure to toxins causes acute and long-term exposure to chronic poisoning. Clinical conditions of acute poisoning appear very quickly, while chronic poisoning manifests itself gradually, is barely noticeable and manifests itself very often after a few years. Toxicological conditions in organisms as a result of exposure to pesticide residues, heavy metals, various chemical food additives, etc., most often manifest as chronic poisoning.

Toxins produced by living organisms (bio-toxic hazards) can be divided according to the organisms that create them into:

- Zootoxins – poisons of animal origin
- Phytotoxins – poisons of plant origin
- Bacteriotoxins – toxins produced by bacteria that are divided into:
  - Endotoxins – produced in bacteria (e.g., *Salmonella*)
  - Exotoxins – secreted by bacteria (e.g., aflatoxin, botulinum toxin)
  - Mycotoxins – produced by fungi

Today's life cannot be imagined without the daily use of chemicals. They are used in the form of medicines for therapy, in the form of pesticides they are used in agronomy, in industry they are based on all chemical syntheses and production, in households cleaning agents enable quality life, and every day we are in contact with countless substances present in the products, for care, hygiene, and medical and decorative cosmetics. In addition, we are surrounded by countless poisons of natural origin, such as plants (poisonous plants and mushrooms), animals (poisonous snakes, spiders, and other animals), and marine (poisonous fish).

As a consequence of daily exposure, a large number of acute and chronic poisonings occur every day, which are already gaining momentum on a modern epidemic, and according to WHO data, poisonings rank third among the most common causes of death in the world immediately after.

1.1.3 "Specific Environment": Definition and Aspects

Anthropogenic pollutants can be defined as any chemical that is unknown to the natural ecosystem and that is released into the natural ecosystem through human activities and can cause undesirable, direct, or indirect effects on human and/or other organisms and on the natural physical, chemical, and biological balance and environmental processes. Anthropogenic pollutants are often produced and used for rapid industrialization in agriculture and industries (Binetti et al. 2008). The discharges of these harmful anthropogenic pollutants have led to local and global environmental degradation when they accumulate in air, water, sediments, soils, and biota, including humans (Lodeiro et al. 2016). This condition creates special and specific conditions in certain segments of the environment. The newly created environment for the living organism can lead to toxicity (if the concentration of certain chemicals is high), or a specific condition is created to which the organisms adapt. Adaptation in the biosphere also leads to modification of some physiological mechanisms in organisms, and they are evaluated over time. In the last three decades, most of these compounds have been hydrophobic organic compounds, attracting the attention of a large number of studies, due to their presence in the environment and the tendency for bioaccumulation. "Hydrophono" means low water solubility, which is a major factor influencing the future of hydrophobic organic compounds in the environment (Binetti et al. 2008).

The future and behavior of organic pollutants in the soil are influenced by soil characteristics, compound properties, and environmental factors, such as temperature and troughs. Possible future of soil pollutants include groundwater leakage, biodegradation, aeration, binding to soil solids, and transfer to organisms. Soil can act as a "reservoir" of anthropogenic organic pollutants in environmental systems due to the fact that the soil has the ability to greatly reduce anthropogenic organic pollutants (Mocarelli 1992). These processes include absorption-desorption in the soil-water interaction, accumulation of plants from the soil solution in the root-soil-rhizosphere interaction, and accumulation of soil microbes from the soil solution in the soil-microorganism interaction. The "storage" of organic pollutants in the soil is controlled by absorption and sequestration of the soil solution, while the "discharge" is regulated by desorption in the soil solution and accumulation and degradation of the plant and soil microorganisms.

Each shift of the natural balance of distribution of biodegradable components in the environment leads to the creation of a specific state in different parts of the environment. Physico-chemical and biological processes and interactions are intensified specifically in conditions of anthropogenic influence on the natural balance of organic and inorganic compounds of the biosphere (Gomes et al. 2017).

Of particular importance are the interactions of soil-water-air-organisms. Diffusion is the only mode of molecular transmission in hydroscopic and capillary waters (Altenburger et al. 2013). In general, the transfer of the organic compound from the soil particles in other phases, such as plants and microorganisms, is controlled by dissolved species and concentrations of the compound in soil environments where there is water (Altenburger et al. 2013). This is due to the fact that the particles in the soil are always surrounded by aqueous films permeating their surface which has a certain polarity and the bisexual water molecules (Altenburger et al. 2013). Furthermore, the nutrition of soil plants and organisms, such as soil bacteria, depends on organic and inorganic compounds dissolved in soil water (Altenburger et al. 2013). Therefore, the distribution of the organic compound in the particles in the soil, air, plants, and microorganisms and their transfer between these phases largely depend on the different physico-chemical and biological interfacial relations related to the water in the soil. These processes include absorption-desorption in the particle-water system, release into the soil air through evaporation of soil water into the water-air system, accumulation of plants from soil water into the root-water system, and accumulation of soil microorganisms from water into microbiological-soil system. The distribution of the organic molecule in these interfaces is in a dynamic state. The statistically obvious balance between the concentrations of the organic compounds in the soil particles and the other phases, such as the water in the soil, can finally be achieved, which helps to understand the interfacial behavior of the organic compounds and their locations in different soil compartments. Although pollutant concentrations in soil, water, and other phases in natural systems may shift from equilibrium, statistical equilibrium data can serve as a necessary guide for the direction of pollutant movement at a given point in time, and this is most likely due to previous pollution. This data is very important to explain whether individual ecosystems (such as soil particles) function as a disposal site (receiving pollutants) or as a source (releasing pollutants) under certain conditions.

Understanding the behavior of organic compounds in these interfaces can help assess the identity, distribution, transfer, bioavailability, and environmental risks of these compounds in the environment. It may also be useful to develop techniques and/or to choose a technique for changing the distribution and transfer of organic compounds in these interfaces which will have useful results (Gariazzo et al. 2005). For example, a plant or microbe with a high capacity to accumulate and degrade organic compounds can be selected and grown to remediate contaminated soils. Furthermore, an additive can be added to the contaminated soil to accelerate the absorption of organic compounds into the soil particles in order to sequester it in the soil and to reduce their concentration in the soil water and its accumulation by the roots, which will contribute to greater food safety produced from these plants that grow on contaminated soils (Beliaeff and Burgeot 2002).

1.2 Environmental Degradation vs. Technological Development Progression

There have been a number of encouraging trends in environmental protection in the last decade: greenhouse gas emissions are declining; the share of renewable energy sources is increasing; certain indicators of air and water pollution show significant improvements worldwide, although this does not always result in with good air and water quality; and material use and waste generation, while still growing, are growing at a slower rate than the economy.

A number of environmental issues reflect key features of systemic risk:

- Many of the environmental problems, such as climate.
- Changes and biodiversity loss are related and have a complex and often global character.
- They are closely related to other challenges, such as the unsustainable use of resources, which extend to the social and economic sphere and undermine the services of the ecosystem.
- Environmental problems are becoming more complex and intensified, even related to social problems, which increases the risks that arise from them.

1.2.1 General Climate Change Issue

The global climate has been extremely stable for the last 10,000 years and has provided the conditions for the development of human civilization, but today there are clear signs that the climate is changing (Conway et al. 1988). This is widely recognized as one of the most significant challenges facing humanity. Measurements of global atmospheric greenhouse gas (GHG) concentrations show an evident increase over the pre-industrial period, with carbon dioxide (CO_2) levels well above the natural range of the past 650,000 years (Conway et al. 1988). The increase in GHG emissions is largely due to the use of fossil fuels, although deforestation, changes in the purpose of land, and agriculture have a significant but smaller contribution.

The main sources of GHG anthropogenic emissions globally are the burning of fossil fuels for electricity generation, transport, industry, and households – which together account for about two-thirds of total global emissions. Other sources include deforestation, which accounts for one-fifth, agriculture, waste disposal, and the use of industrial fluorinated gases. In general, in the EU, energy consumption – the production and consumption of electricity and heat in industry, transport, and households – accounts for almost 80% of GHG emissions (Longhetto et al. 1997).

On the one hand, emissions grew due to a series of factors, such as:

- Increase in the production of electricity and heat in thermal power plants, which grew both in absolute terms and in comparison with other sources
- Economic growth in the processing industries
- Growing demand for passenger and freight transport
- Growing share of road traffic compared to other modes of transport
- Growing number of households
- Demographic changes in recent decades
- On the other hand, emissions dropped in the same period due to factors such as:
- Improvements in energy efficiency, especially among industry and energy end users
- Improvements in vehicle fuel efficiency
- Better waste management and greater utilization of gas from landfills (waste sector achieved the largest relative reductions)
- Reductions in agricultural emissions
- Transition from coal to less polluting fuels, especially gas and biomass for electricity and heat production

Climate change is predicted to play a significant role in biodiversity loss and jeopardize ecosystem functions. Changing climatic conditions are responsible, for example, for observed changes in the distribution of a number of plant species, north and up the hills. The combination of the rate of climate change and habitat fragmentation is likely to impede the migration of many plant and animal species and may lead to changes in species composition and further decline in biodiversity in Europe. Changes in seasonal occurrences, flowering dates, and agricultural growing seasons are observed and projected. Phenological changes have also increased the growing season of several crops in northern latitudes in recent decades, favoring the introduction of new species that were previously unsuitable. At the same time, there is a shortening of the growing season in southern latitudes. It is anticipated that such changes in crop cycles will continue, with the potential for serious impacts on agricultural practices (Carvalho 2017).

Similarly, climate change is expected to threaten aquatic ecosystems. Surface water heating can have several effects on water quality, hence their use by humans. These include more likely algae blooms and freshwater species to move north, as well as changes in phenology. Also, in marine ecosystems, climate change may affect the geographical distribution of plankton and fish, for example, a change in the flowering time of phytoplankton in spring will put additional strain on fish stocks and related economic activities.

Information on the effects of climate change on soil and the various associated feedback effects is very limited, while changes in soil biophysical character are probably due to projected rising temperatures, variability in intensity and frequency of precipitation, and more severe flooding. Such changes can lead to a reduction in soil organic carbon reserves and a significant increase in CO_2 emissions. Projected increased variations in pattern and intensity rainfall are possible and make soils more susceptible to erosion. The corresponding risks are largely dependent on human behavior and the quality of health services. Furthermore, the outbreak of a number of vector-borne diseases, as well as water-related diseases, may become more frequent with rising temperatures and frequent extreme events.

1.2.2 Biodiversity Loss: Degradation of the Natural Capital

"Biodiversity" includes all living organisms found in the atmosphere, on land, and in water. All species play a role and make up the "material of life" on which we depend: from the smallest bacterium in the soil to the largest mammal in the ocean. The four basic constituent blocks of biodiversity are rows, species, habitats, and ecosystems (Mishra and Dhar 2004). Therefore, the preservation of biodiversity is fundamental to human well-being and the sustainable provision of natural resources. Furthermore, biodiversity is tight intertwined with other environmental issues, such as climate change adaptation or human health protection. Biodiversity is strongly influenced by human activities, including agriculture, forestry, and fisheries, as well as urbanization (Dukes and Mooney 1999). Huge areas of land are being cultivated globally, most forests are being exploited, and natural areas are increasingly being fragmented with urban areas and infrastructure construction. The marine environment is also severely endangered, not only by unsustainable fishing but also by other activities, such as oil and gas extraction into the sea, sand and gravel mining, transport, and wind farms at sea.

Utilization of natural resources usually leads to disruption and change in the diversity of species and habitats. In this context, the extensive agricultural practices, as in the traditional agricultural areas of Europe, contribute to a greater diversity at the regional level, compared to what would be expected in strictly natural systems (Dukes and Mooney 1999). But over-exploitation can lead to the degradation of natural ecosystems and eventually the extinction of species. Examples of such recurrent environmental effects are the decline of commercial fish stocks due to overfishing, reduced pollination due to intensive agriculture and reduced water retention, and increased flood risks due to plateau destruction (Dukes and Mooney 1999; Forman and Alexander 1998).

Land conversion leads to loss of biodiversity and degradation of soil functions. In general, urban areas have expanded further at the expense of all other land cover categories, with the exception of forests and water bodies. Urbanization and expanded transport networks are fragmenting habitats, making animal and plant populations more vulnerable to local extinction due to disrupted migration and fragmentation. These changes in land cover affect ecosystem services (Forman and Alexander 1998). Soil characteristics play an essential role here, as they affect water, nutrient, and carbon cycles. Soil organic matter is a major terrestrial carbon absorber and is therefore important for climate change mitigation. Peat soils are the highest concentration of organic matter in all soils, followed by extensively managed grasslands and forests: therefore, the conversion of these systems leads to carbon losses in the soil. Loss of these habitats is also associated with reduced water retention capacity, increased risk of flooding and erosion, and reduced attractiveness for outdoor recreation (McCarty 2001).

Forests are being exploited intensively: the share of the old constituents is critically low. Forests are essential for biodiversity and the realization of ecosystem services. They provide natural habitats for flora and fauna, protection against soil erosion and flooding, carbon sequestration, and climate regulation and have great recreational and cultural value. The forest is the dominant natural vegetation in Europe, but the remaining forests in Europe are far from intact. Most of them are intensively exploited. Exploited forests usually lack large amounts of dead wood and older trees as habitat for the species and often show a high percentage of non-native tree species (McKinney 2002).

Agricultural areas are shrinking, but management is intensifying: species-rich grasslands are in decline. The concept of ecosystem services is probably most obvious to agriculture. The primary purpose is to provide food, but agricultural land also provides many other ecosystem services. Traditional agricultural landscapes in Europe are a great cultural treasure, attract tourism, and offer opportunities for outdoor recreation. Agricultural soils play a key role in the circular movement of nutrients and water (McCarty 2001).

Agriculture is characterized by a double trend: great intensification in some regions and abandonment of land in others. Intensification aims to provide higher yields and requires investment in machinery, drainage, fertilizers, and pesticides. It is also often associated with simplified crop rotation. When socioeconomic and biophysical conditions do not allow it, agriculture remains extensive or abandoned. These movements are driven by a combination of factors, including technological innovation, political support, and international market movements, as well as climate change, demographic trends, and lifestyle changes. Concentration and optimization of agricultural production have major consequences for birds and butterflies from agricultural habitats (McCarty 2001).

Inland and freshwater ecosystems are still under pressure despite reduced pollution loads. In addition to the direct effects of land conversion and utilization, human activities such as agriculture, industry, waste generation, and transport cause indirect and cumulative effects on biodiversity – mainly through air, soil, and water pollution. A wide range of pollutants, including excess nutrients, pesticides, microbes, industrial chemicals, metals, and pharmaceuticals, ends up in soil or in groundwater and surface water. Atmospheric deposition of substances that cause eutrophication and acidification, including nitrogen oxide (NOx), ammonium plus ammonia (NHx), and sulfur dioxide (SO_2), complements the cocktail of pollutants. The effects on ecosystems range from damage to forests and lakes with acidification; habitat disturbance due to nutrient enrichment; algal blooms caused by nutrient enrichment; neurological and endocrine disorders in pesticide species, steroid estrogens, and industrial chemicals such as PCBs; and neurological and endocrine disorder in species (Ellis 2011).

Maintaining biodiversity globally is essential for humans. Biodiversity loss ultimately has far-reaching consequences for humans through impacts on ecosystem services. Large-scale processing and drying of natural systems increase carbon emissions into the air while reducing carbon and water holding capacity. Increased leakage rates, combined with increased rainfall as a result of climate change, are a dangerous cocktail experienced by a growing number of people in the form of severe flooding (Ellis 2011).

Biodiversity also affects well-being by providing opportunities for recreation and beautiful scenery, a relationship that is increasingly recognized in urban design and spatial planning. Perhaps less obvious but equally important is the relationship between patterns of distribution of species and habitats and vector-borne diseases. Invasive non-native species in this regard may pose a threat. Their spreading capacity and the potential to become invasive increase with the globalization of trade, combined with climate change and the increasing vulnerability of agricultural monocultures (Ellis 2011).

1.2.3 Natural Resources and Waste

The overall impact of resource use globally continues to grow. The world relies heavily on natural resources to guide its economic development. There are growing concerns about the sustainability of these models, especially the implications for the use and overuse of natural resources. The assessment of natural resources and waste in this chapter complements the assessment of biotic natural resources in the previous chapter by focusing on material and often non-renewable resources, as well as water resources. A look at natural resources in terms of life cycle reveals several environmental problems related to production and consumption and combines resource use and waste generation. Resource utilization and waste generation are specific impacts on the environment, but the two issues share many of the same drivers – largely related to how and where we produce and consume goods and how we use natural capital to sustain patterns of economic development and consumption.

Waste management continues to shift from waste to recycling and prevention. Every society with a history of rapid industrial development and consumption faces the issue of sustainable waste management, and this issue continues to raise significant concerns; worldwide, it is determined to reduce waste generation, but it fails. Trends in those data streams for which data are available indicate the need to reduce waste generation in absolute terms to reduce further environmental impacts.

Waste policies can primarily reduce three types of environmental pressures: emissions from wastewater treatment plants, such as methane from landfills; the effects of the extraction of primary raw materials; and air pollution and greenhouse gas emissions from energy use in production processes. Although recycling processes in turn also have environmental impacts, in most cases, the overall impacts avoided by recycling and utilization are greater than those occurring in recycling processes. Waste prevention can help reduce environmental impacts during all phases of the resource life cycle. Although prevention has the highest potential to reduce environmental pressures, policies to reduce waste generation are rare and often not very effective. For example, emphasis was placed on the diversion of biowaste, including food waste from landfills. But more could be achieved by treating the entire food production and consumption chain to prevent waste and thus contribute to the sustainable use of resources, soil protection, and climate change mitigation.

1.3 Basic Principles of Analytic Model Design

Scientific work is a systematic and logical study that is subject to a complex methodology composed of many principles and methods, aimed at finding and revealing facts and truths about given questions, phenomena, and processes. Every science in its scientific research applies certain procedures and methods. Accordingly, anyone who wants to be interested in science and be successful in their scientific work will need to be well acquainted with the methodology of scientific work while respecting the methods and rules used in the field of his scientific interest. In the last few decades, the methodology has significantly developed as a special scientific discipline. Namely, the methodology is a way – a method that uses certain means and paths with the help of which the scientific workers come to certain knowledge. The subject of the methodology in itself encompasses many principles of general logic – the science of the rules and the path of correct thinking.

The subject and function of the methodology are closely related with epistemology (theory of scientific consciousness). However, the methodology does not only study the path and the means for solving certain problems, but it is also oriented toward the study of the problems related to acquiring new knowledge in order to increase the quality of the scientific system.

In the subject of every science, and hence the methodology itself, it cannot exist without the following basic characteristics of scientific consciousness: objectivity, certainty, verifiability, precision, systematicity, composition, and totality:

- Objectivity implies the absence of bias, truthfulness, neutrality, fairness, active attitude of the scientist, discipline in research, and indifference.
- Security implies perseverance and dedication to work to achieve goals.
- Verifiability and confidentiality imply consistency and argumentation toward the research.

- Precision implies the effort of the researcher to be correct and persistent at all stages in the research process.
- Systematization implies the organization of the researcher throughout the entire research process.
- Composition implies continuous application of scientific methods in the scientific process while actively adhering to the subject, purpose, and structure of the research.
- Totality or generalization implies a tendency in the research process to discover certain phenomena and relations that are in favor of scientific consciousness and applicability.

Most scientific research is subject to the subjective opinions and assumptions of a number of scientists. Objectivity has always been eroded by the subjective opinions and assumptions of a scientist who represents the so-called absolute skepticism, that is, absolute mentioning in everything and everything, that is, in every scientific consciousness. However, in science, it is allowed and so on. Methodical skepticism basically means criticism of every piece of information in science, of every move of science, etc., and this is in fact one of the important features of the scientific way of thinking. If this skepticism is realized in this way, as doubt in everything until the reasons for the doubt are discarded, then we can freely say that methodical skepticism is strong and ingrained in science.

Generally, in scientific work, there are three basic groups of problems: logical, technical, and scientific strategic. Logical problems are the formulation of scientific concepts, hypothesis, and analysis. Technical problems are all means used for the collection of scientific material and research, the use of tools, and the arrangement and processing of data. Strategic problems are all the conclusions that are aimed at solving certain problems in order to find new scientific truths and knowledge. In every work, even in the scientific research, the subjects themselves build their own method of work, but before that, it is necessary to use the methods developed and used by other scientific workers. Consequently, the application of valid and developmental methodologies in scientific research is essential.

1.3.1 Matrices Selection Criteria

Most often, research conducted to examine the quality of the environment is subject to a multidisciplinary approach. Of course, this also refers to the methodological approach to the research. There are more than 30 unified research methodological approaches. When setting up an experimental model for the analysis of environmental segments, the methods of analysis and synthesis are generally applied and used.

Analysis is a thought, theoretical, and practical process that breaks down complex material or intellectual wholes, processes, phenomena, and objects into constituent general and specific parts or aspects, in order to describe and determine how they affect the whole. Analysis is a critical assessment and at the same time a search for a connection between causes and consequences and reaching conclusions by dividing or breaking down the whole of its components. Several activities and techniques are used in making the analysis. In recent years, cluster or group analysis has often been used, which is a multivariate technique of statistical analysis based on the efforts to determine, identify, and analyze relevant homogeneous objects or variables. Analysis and synthesis in scientific research are always together, and therefore, we can treat them as a single scientific method, analytical-synthetic method.

When it comes to monitoring natural and anthropogenic phenomena in the environment, the application of comparative methodology should certainly be pointed out. It is a method that applies a comparison of the same or related facts, phenomena, processes, and relationships, i.e., determines their similarity and diversity. This method allows researchers to enrich their knowledge and come to new conclusions. The comparison between two things, two phenomena, and two events moves so that first their common things are determined and then they are what distinguish them. The comparison should emphasize what is common and what is different, and it also shows the cognitive role of this method. In this method, words that have a purpose for a certain metaphorical meaning can be applied. Namely, through pictorial expressions, various words – most often adjectives – are presented to us that should be known to us, so that the unknown will be compared to something similar to it and known to us. Important features and goals of this method are identification of common or similar things, phenomena, and forms that are explored; setting the basic hypothesis; testing the hypothesis and its specifics; systematic comparison; and classification of new phenomena in the composition. Objectives are defined by describing the structure, functions, and behavior of parts as common or different; classification of newly known things, phenomena, and their systematization; and detecting or monitoring the development, evolution, strategy, progress, or lag in the development of defined – certain things and phenomena.

Statistics is a tool, a scientific method for studying atypical phenomena expressed in numbers. Statistics is one of the most applied methods in scientific research work in all scientific areas, fields, areas, and disciplines and is therefore known as a general scientific method. Statistics as a scientific method is evolving very fast, so that 28 new statistical procedures-methods are constantly appearing (Charou et al. 2003). These new statistical procedures are applied in certain scientific areas and disciplines. The modern scientist needs knowledge of the statistical method in order to easily collect data, process it, and based on that come to certain conclusions. From the numerous statistical procedures applied in various scientific fields and disciplines, we will mention analysis of attribute and geographical sequences; analysis of numerical sequences (arithmetic mean, median, modus, moment, measures of dispersion and asymmetry, measures of roundness); time series analysis (indices, mean value, time series); theoretical distribution (normal, binomial, Student's, Posson's, and hypergeometric distribution); sample method; graphical representation of statistical data histograms (rectangular or bar chart; structural circles; linear diagram; cartogram; pictogram graph with pictures, dots, or other signs; statistical maps; etc.); and correlation (linear, correlation, curvilinear, partial correlation).

Metric models allow estimation of quantity, determination of variables, development projections, analyses of economic cycles, and movement of supply and demand, whereas the simulation method implies the application of modern calculators and theoretical behavior of phenomena and processes in reality in order to find the most favorable possible solution.

Modeling is a systematic research procedure through which real or thought models are made, i.e., models of sketches, objects, mathematical formulas, etc. (Gilbert 1987). The model is a cognitive dialectical process of the movement of cognition from practice to theory and from theory to practice. The object of modeling can be any phenomenon, i.e., any object, physical or organic. The structure of modeling is made up of four factors: passive objective factors (subject of modeling), active subjective factors, means or tools, and position in objective reality and conditions. Therefore, a model can be any model, sketch, and picture, such as city plan, map, various miniature or real model, organizational chart, etc.

1.3.2 Experimental Design

An experiment is a planned, organized procedure of controlled observation and checking of a law, process, or phenomenon that is examined under precisely certain conditions, which had the purpose of detecting unknown factors, properties, relationships, processes, phenomena, objects, laws, and the like. The experiment is a scientific method that connects them theoretically with practical knowledge. The experimental method is often used in combination with other scientific methods such as the observational experiment. The basics of the experimental method are the experimenter, the subject of the experiment, the means, the instruments, procedures, operations, setting, processing and testing of the hypothesis, obtained results, explanation, analysis and presentation of results, setting new higher goals, hypotheses, new experiment, new results, etc.

Investigations of abnormalities and distributions in the environment should be based on a well-placed experiment. The observation model usually provides the general screening of the situation in the environment. This is how the framework for setting theoretical hypotheses is defined. These hypotheses most often refer to the so-called micro-environments, in which the abnormal occurrence, distorted natural distribution of a given variable, or the content of certain essential components occurs (Dudoit et al. 2003). Observation in order to be scientific must be more objective, conscious, complete, precise, strict, organized, systematic, persistent, etc.

Monitoring is always a cheaper and more efficient methodology when it comes to monitoring a certain phenomenon in the environment. Monitoring, on the other hand, allows research to be time-limited. On the other hand, this observation approach allows the detection of phenomena that may not be foreseen in the hypothesis at the beginning of the research. Additionally, monitoring is an in vivo experiment that should reflect the actual interactions between the variables that are subject to monitoring. Also included are environmental factors that directly and indirectly lead to changes in the observed processes. In this way, the researcher can perform multi-factor dependency analysis, as well as determine the degree of influence of each individual factor.

1.3.3 Chemical Analysis

Environmental research, especially research on environmental degradation processes and their consequences, involves the chemical characterization of a huge number of chemical components of an organic and inorganic nature. They can include qualitative and quantitative determination. Nowadays, rapid technological development has ensured the production of fast, modernized, and effective qualitative and quantitative automatic instruments, which enable rapid qualification and quantification of the chemical composition of any environmental medium.

Mass spectrometry (MS) is a method used for qualitative and quantitative chemical analysis. An important feature of mass spectrometry is its high sensitivity. The mass spectrometer is an instrument in which the examined sample is ionized and the generated ions are separated under the action of a magnetic field and are registered according to the values of the masses (Brack et al. 2019). More precisely, the mass spectrometer determines the ratio of mass and charge of the ion (m/e), but most ions have only a single charge ($e = 1$), so the term "mass" is often used and essentially means the ratio m/e. The first constructed instruments were called mass spectrographs because the registration of ions was done on a photographic plate. Later the electrical ion detection system was perfected and the name was changed to a mass spectrometer. There is a difference in registration between the two devices: in the spectrograph all ions are registered at once, while in the spectrometer one-by-one type of ions is achieved by continuous change of the magnetic field. In the first half of the last century, mass spectrometry developed much more slowly, due to the imperfection of the instrument as well as due to the high cost. Mass spectrometry achieved a rapid rise in the period 1995–2005. A new ionization source under atmospheric pressure was developed, existing analyzers were suitable, so a hybrid instrument was developed with a combination of analyzers. The analyzer based on the new concept was further used to develop new applications.

The first step in analyzing a sample by mass spectrometry is to create a gas phase of ions from the sample for analysis, for example, by electronic ionization:

$$M + e^{-} \rightarrow M^{\cdot +} + 2e^{-}$$

This $M^{\bullet+}$ molecular ion is further subjected to additional fragmentation. Since it is a radical cation with an odd number of electrons, it is possible to fragment it to obtain a radical ($R^{\bullet}$) and an ion with a positive number of electrons (EE^{+}) or a molecule (N) and a new cation with an odd number of electrons ($OE^{\bullet+}$):

$$M^{\cdot+} \rightarrow EE^{+} + R^{\cdot}$$
$$M^{\cdot+} \rightarrow OE^{\cdot+} + N$$

Both types of ions have different chemical properties. All these ions are separated in the mass spectrometer according to their mass/charge ratio and are detected according to their concentration (number). In this way, the mass spectrum of the molecule is created and constructed. The mass spectrum is constructed as a graph of the ion concentration of ions versus the mass/charge (m/z) ratio. Most positive ions have a charge that corresponds to the emission of only one electron. When analyzing larger molecules, several different charged ions can be obtained. The ions are separated and detected according to the m/z ratio. The total charge of the ions will be represented by the parameter q, the charge of the electron e, and the number of ions z as:

$$q = ze, \quad \text{where } e = 1.6 * 10^{-19}\,\mathrm{C}$$

In the graphical representation of the mass spectrum, the mass/charge ratio, commonly denoted as m/z, is represented on the x-axis. When m is given as a relative mass and z charge, and both quantities are given as dimensionless values, m/z is used to determine a dimensionless quantity. Generally, in mass spectrometry, the charge is determined by multiplying the elemental charge or the charge of one electron by its absolute value ($1.6*10^{-19}$ C). Mass is determined by the atomic unit of mass ($1u = 1.660540*10^{-27}$ kg). As mentioned earlier, the physical quantity measured in mass spectrometry is the mass/charge ratio. When mass is expressed in atomic mass units (*u*) and charge is expressed in basic units of charge (*e*), then the mass/charge ratio gets a unit of measurement *u*/*e*. To simplify things, a new Thomson unit has been proposed, with the symbol Th. The definition for this unit of measurement is:

$$1\ \mathrm{Th} = 1\ u/e = 1.036426 \cdot 10^{-8}\,\mathrm{kgC^{-1}}$$

Both the atomic mass units *u* and Da have a precisely defined and fundamental definition:

$$1\ u = 1\ \mathrm{Da} = 1.660554 \cdot 10^{-27}\ \mathrm{kg} \pm 0.59\ \mathrm{ppm}$$

Depending on the main purpose of the research, the appropriate units are used, i.e., when we are faced with medium isotope masses, as is usually the case in stoichiometric calculations, Da is more suitable, whereas in mass spectrometry, when masses refer to the basic isotopes of the elements, mass is expressed in u mass units.

There are different ways to define, and thus calculate, the mass of an atom, molecule, or ion. In stoichiometric calculations, the average mass is usually used, calculated from the atomic mass obtained as the average of the atomic masses of the different isotopes of each element in the molecule. In mass spectrometry, the nominal mass or monoisotopic mass is most commonly used. The nominal mass is calculated using the mass of the dominant isotope of each element, rounded to the nearest integer value of the mass number, also known as the "number of nucleons." But real isotope masses are not exact integers. They vary poorly in relation to their summable values of their constituents such as protons, neutrons, and electrons. These differences, called "mass defects," are equivalent to the binding energy that holds these particles together.

Therefore, each isotope has a unique and characteristic defect mass. Monoisotopic masses, which are included in the calculations of these mass defects, are calculated using the exact mass of the most common isotope for each constituent element in a molecule. The difference between the mean mass, the nominal mass, and the monoisotopic mass can be several Da units, depending on the number of atoms and their isotopic composition. Mass spectrometers must operate under high vacuum (low atmospheric pressure). This is necessary because of the ability to allow ions to reach the detector without colliding with molecules of other gases (Krauss et al. 2010).

The development of mass spectrometry has been directed according to the requirements of industry, science, and routine laboratories for obtaining greater sensitivity and multifacetedness in maintaining the level of traces and ultratracks. The development trend of this technique for quantitative and qualitative analysis was also aimed at minimizing the sample for analysis with improved sensitivity and lowering the limit of detection and quantification. These features were accompanied by the need to increase the speed of analysis. Minimizing the sample for analysis also meant reducing the volume of waste in order to develop a "green chemistry" approach. The development of new techniques for the application of mass spectrometry as a quantitative technique was also aimed at proving the accuracy and precision of analytical data.

1.4 Generating Data Matrix (What to Do with Informations?)

1.4.1 Generating of Hypotheses: Accurate Research Pathway

Different types of hypotheses stand out in the theory: ad hoc hypothesis (preliminary hypothesis), working hypothesis, auxiliary hypothesis, and scientific hypothesis. Hypothesis setting has a great importance in scientific research work. Hypothesis setting shows the extent to which we have mastered the material we are researching and is a sure orientation in our further work. The formulation of the working hypothesis is an assumption for which we have certain data useful at a certain stage of our research. The auxiliary hypothesis serves the basic or working hypothesis and as such helps the working hypothesis to be easier to verify. However, the main significance of the hypothesis is in its guiding role in research. It can impose new facts and information on us, put us in a position to check it and prove it, or put us in a position to discard certain insignificant parts or supplement it with new knowledge, to impose on us the need for further research that may be against our hypothesis, or, in the last resort, to change the original hypothesis and formulate a new hypothesis.

The process of researching and gathering information can take place over a shorter and longer period of time. In the process of information processing, the researcher encounters the application of several scientific research methods and in doing so applies appropriate data processing techniques. The process of processing the topic is followed by collecting, selecting, processing, accepting, and putting the information in the topic.

The hypothesis is theoretically a well-reasoned and empirically verifiable answer to the question with which the problem is expressed. The hypothetical framework of research through the system of hypothesis (general, specific, and individual), their variables, and the relationship between them and the important indicators report basic assumptions about the subject of the research and provide a full understanding of the essence of the research project. In order to overcome the difficulties of hypothesizing, a set of rules or norms for setting appropriate hypothesis are formulated. These are the following rules:

- The hypothesis must be a logical and scientific-theoretical established assumption with a cognitive value response to a significant issue.
- The hypothesis should be appropriate to the subject of research.
- The hypothesis must be specific enough to determine the phenomenon under study.
- The hypothesis must be general enough.
- The hypothesis must be clear and linguistically clear and formulated as accurately as possible.
- The empirical hypothesis must have certain empirical one's references, i.e., it must be based on some empirical data and facts.
- The hypothesis must be theoretical and in the empirical sciences practically verifiable.
- The hypothesis should be based on the scientific theory of that area of occurrence to which the hypothesis refers.

Hypotheses must be appropriate and symmetrical in the operational determination of the research subject, which means that they should be promoted to the level at which the research subject is developed. All these concepts must be explained in the operational determination of the subject of research. The special hypotheses refer to special conditions or structural factors of the subject of research that are identified in the operational determination of the subject of research. The individual hypotheses are referring to individual terms or sub-terms of certain structural factors of the subject of research which are listed in the operational determination of the subject of research.

Variables are necessary, constituent factors of any hypothesis. "Variables are derived or taken from the operational determination of the subject of research." A basic feature of each variable is the ability to replace its meaning. Therefore, every characteristic of any phenomenon is variable where we see qualitative or quantitative differences that occur in it. In any research, there are two types of variables:

- Independent variables
- Dependent variables

Independent variables are the basis for interpreting, explaining, or describing dependent variables. "Independent variables are those variables that exert some influence, they are the causes of a phenomenon, and dependent variables are those that are influenced or which are the consequences of such influences. Therefore, what is defined in the dependent variable depends on the independent variables. Independent variables have a constant and permanent impact, but dependent variables change depending on that impact. Each independent variable contains process-causing indicators. Thus, the indicator is contained in the variables and affects the process, the occurrence."

The variable occurs as a consequence of certain reasons and as a cause for certain consequences. Therefore, the researcher cannot arbitrarily set the variables, but must derive them from the operational determination of the object of research, which begins the derivation of hypotheses. According to the content, there are qualitative and quantitative variables. Qualitative variables express variable properties, forms, relationships, phenomena, or factors of the phenomenon under investigation. Quantitative variables refer to the dimensions, quantity, frequency, and other quantitative provisions or characteristics of the occurrence or occurrence factors under investigation.

The operationalization of the research object implies that for each hypothesis, indicators are defined, i.e., types of data that the research can achieve, which are of such a quality that they can confirm or deny the hypothesis.

In the theory of methodological literature, the perspective of conceptualization of research as a general idea, a general plan of upcoming research is widespread. In the context of conceptualization are all stages of research – from topic selection to reconceptualization. Therefore, both the research project and the project work are part of conceptualization, i.e., reconceptualization. The manner of preparation of the project for scientific research and its role in the research, as well as its other characteristics, require an explanation of the relationship between conceptualization – reconceptualization and research project.

Conceptually, conceptualization means the construction, elaboration, and development of the most general scientific ideas for supposed, possible research. This most general idea starts with a general idea of observed relationships, a recorded problem from a scientific and practical point of view, i.e., scientific-theoretical or empirical problem. Based on the formulated idea, a preliminary definition of the research problem follows, which leads to closer and more complete information about it. The existing scientific and empirical knowledge that we have gained suggests the possibility of researching the defined problem. So, the concept of research is formed which is expressed in general terms. However, valid scientific research, as a rule, cannot be carried out on the basis of general provisions of the concept, but requires deeper and more detailed scientific analysis.

The main function of research planning is to synchronize all activities, participants, and resources in a given space and time. Therefore, it can be summarized that the research plans set out include: a) temporal and spatial definitions of the research subject, b) research method, c) draft scientific ideas, e) the real situation and the available resources for the realization of the research activity. The result of the planning process is an operational research plan. The task of this plan is to answer the following questions: who, what, when, where, how, and with what consequences. The operational research plan can be discussed in a narrower and broader sense. In a broader sense, the operational research plan includes:

- Methodology of research of the proposed ideas, and in some cases (when researching the factual – current phenomenon) operational research plan includes temporal and spatial definition of the object.
- Time, personnel, and resource plan
- Instrument for data collection and processing

The broader understandings revealed, if they appear to be too broad, include what might be termed a "scientific plan," which includes certain scientific-theoretical and scientific-instrumental solutions contained in the draft scientific ideas and instrumentalization.

1.4.2 Uncertainties

An important element of environmental quality control is the recognition of the displacement of measures that probably do not reflect natural variability but signal a structural cause that requires further investigation. Systemic or specific causes are the result of external (usually anthropogenic) sources, which need to be discovered because they are an integral part of the process and cannot be avoided. The occurrence of these causes is quite unpredictable, accidental, and unsystematic, and it is necessary to take corrective action to remove it from the process. The total variation of the process/change is equal to the sum of the specific (systematic) and random (unsystematic) causes of variation. Thanks to various control techniques, there are many ways to carry out statistical control of changes and abnormalities.

1.4.3 Statistical Models

Environment statistics describe the qualitative and quantitative aspects of the state and changes of the environment and its interaction with human activities and natural events. These mathematical methods are integrative, measure human activities and natural events that affect the environment, and monitor the impacts on the environment and the social responses to environmental impacts.

Environment statistics is a domain of statistics, and it is indispensable for evidence-based policies and decision-making to support sustainable development. Humans use environmental resources for production and consumption, and they return residuals and waste to the environment. As a result of human activities, environmental conditions, natural processes, and the capacity of ecosystems to provide their goods and services all experience change. These changes, in turn, initiate changes in the human subsystem's economic and social processes. Immediate objective of environment statistics is to provide quantitative information about the environment's state and its most important changes over time and across territories. Operationally, the objective and ultimate purpose of environment statistics can be achieved by setting up, strengthening, and sustaining environment statistics programs and units that function on a regular basis in each country, similar to those already operating in economic and social statistics. Environment statistics is a cross-cutting statistical domain with specific complexities. The demand for environment statistics is increasing rapidly everywhere. Environment statistics production requires combined technical expertise on environmental themes, statistical technical capacities, and institutional coordination capacities. By definition, environment statistics are multidisciplinary and cross-cutting, involving numerous stakeholders.

Environment statistics' sources are dispersed, and a variety of methods are applied in their compilation. They are collected through censuses, surveys, and the use of administrative records, but also from specific types of sources such as monitoring stations, remote sensing, and scientific research. The way of collecting data from the specific types of sources differs considerably from the survey techniques employed in social and economic statistics: from the environment to the production of biophysical data describing natural resources and environmental quality.

1.4.4 Quality of Prediction and Model Validation

The interdisciplinary and inter-institutional characteristics of environment statistics and the variety of data producers and users require collaboration among institutions, practitioners, and experts in different fields. To effectively transform environmental information into official environment statistics, the collaboration and coordination of a significant number of actors and institutions is required. Institutions with strong leadership and the skills and resources to facilitate multi-stakeholder processes are required. Thus, institutional strengthening and inter-institutional collaboration and coordination are inherent to environment statistics data production, processing, and dissemination.

Environmental phenomena take place in dispersed manner, constantly over time and across territories, as a fluid set of processes, each with different velocities. At the primary sources, measuring and monitoring requires careful selection. Transforming raw data from primary sources to statistics takes expertise and institutional capacities. Developing countries face budgetary constraints, and the funds allocated to statistics are subject to restrictions. In general, environmental issues are given a lower priority than social and economic statistics. The cost of monitoring systems and remote sensing is falling over time, and some of the primary production of raw data (in monitoring and remote sensing agencies) require the investment, calibration, and use of somewhat costly instruments, not to mention the work of interpreting data (such as satellite images). This means that the country has to invest in developing these monitoring capabilities and also that scientists and highly specialized experts must be hired on a stable basis. Remote sensing in particular also requires the validation of the imagery data with direct observation in the field, thereby increasing the cost of producing environment statistics by this method, relative to the cost of statistics derived from questionnaires, for example, prioritizing environment statistics that are most relevant, critical, or strategic and work progressively on further expansion.

1.5 Basic Statistical Models: Challenges and Atypical Implementations

Environment statistics is also referred to as basic environment statistics, usually voluminous sets of statistics describing the state and trends of the environment and their main components. It includes the human subsystem in its interrelation to ecosystems as a whole. To transform raw data into official statistics requires a carefully tailored process of definition of the types of magnitudes (i.e., aggregates, averages, minimums, maximums, etc.) and its attributes (time, location, coverage, etc.) to be captured by carefully selected variables that are then collected, validated, structured, and described using statistical standards and procedures (more later). Usually the environment statistics series are produced for countries, regions, and the world and disseminated through compendia and databases. Because of their volume, the general public and the decision-makers often require further processing of environment statistics models.

Environmental indicators are a particular type of statistics, requiring a careful selection of individual statistics in order to calculate a composite or more complex measure to depict key elements of processes about the environment. Indicator sets are typically produced to monitor national (and international) policy goals and targets and to enable continued oversee of progress toward sought objectives. Environmental indicators are widely produced as a stand-alone product, but sometimes environmental indicators are part of sets of sustainable development indicators, along with accompanying economic and social indicators. Indicators are powerful measures that are usually disseminated with an accompanying context and explanation. Frequently, environmental indicators are disseminated through reports, brochures, and websites and are widely used in assessment and reports about the state and trends of the environment.

1.5.1 Correlations and Similarity Models: Challenges and Atypical Implementations

The correlation is interdependence and mutual dependence, i.e., mutual connection and dependence, the relationship between two economic phenomena that will be found in causal dependence and conditioning. The correlation can be parallel, i.e., positive and, vice versa, negative. The correlation is positive when the growth of one phenomenon causes the growth of the other (e.g., the growth of the demand causes the growth of the price) or the reduction of one phenomenon causes the reduction of the other. The correlation is negative when the growth of one phenomenon is followed by the fall of the other (e.g., the ratio of changes in price and demand) or, conversely, the reduction of one phenomenon is followed by growth of the other. The intensity of the correlation is measured by the correlation coefficient. The notion of correlation in many ways coincides with the notion of function.

Bivariate analysis is used to perform a comparative analysis of the dependencies between variables (set of values for one variable). Two-dimensional scatterplots are used to display the correlations and identify them visually.

Multivariate analysis is applied to extract dominant associations of variables (Šajn 2006). As a measure of similarity between variables, the product-moment correlation coefficient (r) was applied. There are various rotational strategies that have been proposed (Žibret and Šajn 2010; Šajn 2006).

The purpose of the applied statistical data processing models is to obtain the most realistic model of multi-dimensional variables' distribution system. For variables, i.e., the data set for the content of a given element, for which the applied factor analysis will deposit low values, they will be excluded from further analysis.

1.5.2 Anomalous and Atypical Occurrence

Newer laboratory instruments, i.e., their software, usually write the data in binary files, in accordance with the so-called good laboratory practice. Data written in this way can be more difficult to manipulate, and this is especially important when analyses have potential legal consequences. However, if we put aside such illegitimate manipulations, users occasionally have some requirements that the software of a given instrument did not anticipate or allow. Very often the graphical display of the experimental data is not as required by the user. Sometimes it is also necessary to perform a more detailed analysis or decomposition of peaks (deconvolution). There are a number of statistical packages and tools that enable the processing of raw data obtained from analytical instruments.

In most cases, the data obtained from the instrumental measurements, in addition to the measured signal, also contain certain noises due to certain influences on the measuring instrument. One of the possibilities is to "filter" the obtained data with program tools contained in the software of the analytical tool. Several noise filtering methods have been developed without altering the underlying signal or signal characteristics. Most often, these data filtering methods are based on a statistical model to determine the so-called dynamic mean. Another useful model is the Savitzky-Golay method. This model is based on the principle of inserting a dynamic mean into a polynomial arrangement. Basically, such models represent the normalization of the share of measuring points (carrier variables), where more weight is given to the central data and less to the peripheral ones.

Environmental investigations deal with numerous data. For a large number of variables with high variance in distribution and high standard deviation (which is often the case for the distribution of environmental variables), there is a problem treating all results simultaneously. In such cases, the variables with the largest variance will have the greatest impact on the outcome of the procedure applied (the appropriate statistical method).

Data transformation and/or standardization is used as a possible solution to the problem. Usually, in asymmetric distributions, the results are first transformed using a logarithmic transformation that helps to facilitate homogeneity in the variance. This procedure will emphasize the influence of the variables with high variance. If this is not the desired effect of the transformed data, then standardization is performed.

Transforming data means performing the same mathematical operations on each component of the original data. If the original data is multiplied or divided by a specific coefficient or is repeatedly subtracted or added, then we are talking about linear transformations. But these linear transformations do not change the shape of the data (i.e., their distribution) and therefore do not help normalize the distribution of the data.

It is usually characteristic of data on certain environmental variables whose distributions are log-normal or positively curved. Therefore, it is necessary, before proceeding with their processing, to undergo a transformation first, that is, to normalize their distributions. Logarithmic transformation is widely applied in order to normalize positively curved data distributions. The purpose of applying a particular data transformation is to actually reduce the difference between extreme values. The Box-Cox transformation method was also used to normalize the data (Box and Cox 1964).

Regression analysis is a typical example of a statistical tool that can be widely applied in solving heterogeneous and multiparametric distributions. In the determination of the correlation parameters, for defining the appropriate model, the regression analysis method can be successfully used. It should be borne in mind that regression analysis can be expressed with other dependencies, not just linear dependencies, as well as multiple parametric dependencies.

Factor analysis is an interdependence technique because it looks for a group of variables that are similar in that they “move together” and therefore have great interdependence. When one variable has a large value, then the other variables in the group have a large value. For the effective application of factor analysis, as well as other multivariate interdependence techniques, it is necessary to have a minimal amount of redundancy of variables, that is, the variables at least slightly overlap in their meaning. Thanks to this redundancy, it is possible to discover a pattern in the behavior of variables, that is, the basic idea (factor) by which they are imbued (Žibret and Šajn 2010).

Cluster analysis is a statistical technique used to identify how different entities – variables – can be grouped together for the sake of their characteristics. Also known as clustering, it is a preliminary data analysis tool that aims to sort different objects in a group in such a way that those who belong to the same group have the highest degree of association. The most commonly processed dendrograms or clusters are expressed by distance, Dlink/Dmax (%).

Characteristic vector analysis (PCA) is methodologically very similar to factor analysis, in that it uses the correlation coefficient (r) to isolate new components that do not show dependence on each other. Often the representation of the dependency is given graphically in a two-dimensional system by the two most significant components (vectors). Component analysis provides beside graphical and tabular calculations for easy explanation of graphical representations. This module is classified by a diagonal, symmetric, correlation, or covariance matrix.

1.6 Conclusions and Perspectives

High utilization of resources to feed economic growth exacerbates the problems of securing supply and sustainable yields and managing environmental impacts in terms of ecosystem absorption capacity. A challenge for politics, as well as for science, is how best to measure the environmental impacts that result from the use of resources; several ongoing initiatives aim to better quantify environmental impacts from resource use. Environmental degradation through air pollution, noise, chemicals, poor-quality water, and loss of natural habitat, combined with lifestyle changes, is likely to contribute to significant increases in obesity, diabetes, and cardiovascular and neurological diseases and cancer – all of which pose major health problems for the population. Reproductive and mental health problems are also on the rise. Asthma, allergies, and some cancers associated with environmental stress are of particular concern to children. All this indicates the great need for continuous qualitative and quantitative assessment of the state of the environment. Quantitative analyses and methodologies are multidisciplinary chemometric models that are applicable in almost all areas and enhancements to environmental research. The last decades have seen an intense technological development, which has enabled the launch of very sensitive, fast, accurate, and effective analytical instruments. This development of instrumental analytical models has enabled the chemical characterization of a huge number of pollutants and toxins, which degrade the environment even in very low concentrations.

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Chapter 2

Spatial Series and Multivariate Analysis in Assessing the Essential (Cu and Zn) and Toxic (As, Cd, Cr, Co, Hg, Ni and Pb) Metals Linked with Health Risk and Ecological Effects of Atmospheric Deposition by Using Bryophyte Moss as Bioindicator

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Abstract Toxic metals are among the main threats to environmental pollution as they pose risk to the human health via different exposure pathways and to the environment. This chapter is focused on spatial series and multivariate analysis in assessing the toxic metals linked with health risk (As, Cd, Cr, Co, Hg, Pb, Ni and Zn) and ecological effects of atmospheric deposition by using mosses as bioindicator. It is based on the concentration data onto eight elements in moss samples collected during the 2015 moss biomonitoring survey conducted at the same time with the European moss biomonitoring. The elements under investigation were determined by ICP-AES and CVAAS (only Hg) analytical techniques performed in the Institute of Chemistry, Faculty of Science, Ss. Cyril and Methodius University, Skopje, North Macedonia, and Faculty of Natural Sciences, University of Tirana, Albania. *Hypnum cupressiforme* (Hedw) spp. have been used as bioindicators of trace toxic metals in atmospheric deposition in Albania. The spatial analysis of each

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element performed by a space series model, adapted to the time-series linear model, was used to investigate the spatial distribution of the elements. It may visualize the areas characterized by a high concentration level of the toxic elements, particularly of As, Cd, Cr, Hg, Ni and Pb, that are classified as human carcinogens. Multivariate analysis is used to identify the most probable sources of the elements in moss samples. Significant differences were found in the concentrations of As, Cd, Cr, Co, Hg, Ni and Pb in moss samples, and diverse patterns for different elements were found by presenting diverse geographical variabilities in moss metal concentrations. Good correlations were found between the concentration data and the normalized concentration data that indicate higher effects of anthropogenic sources of these elements compared to the natural one. High Ni and Cr contents were found in all territory of Albania. The highest Ni and Cr contents were found in the eastern part that could be classified as highly polluted zone mostly by geological and anthropogenic emission sources. The elements Cr, Ni and Hg exceeded the corresponding median values of European moss. The spatial analysis of the elements (linear model) is possible to identify the areas with a high concentration of certain metals which could suggest for being under control. Wide ranges of metal concentrations were found for As, Hg, Cd, Pb, Ni, Co, Cr and Zn which are characterized by high variations (CV% > 75%). The highest content of Cr, Ni and Co was found in the eastern part of the country. The anthropogenic factors and soil dust associated with the windblown fine mineral dust particles were pointed as another possibility of local emitting factors affecting the accumulation of these metals in mosses. The median value of CR (1.36×10^{-6}) is higher than the carcinogenic target risk value (1×10^{-6}) and lower than the Cr unacceptable value ($CR > 1 \times 10^{-4}$).

Keywords Atmospheric deposition · Trace metals · Moss biomonitoring · Air quality · ICP-AES analysis · Health risk · Multivariate analysis · Spatial analysis

2.1 Introduction

Air pollution is caused by various natural and anthropogenic processes that cause the growth of various organic and inorganic chemicals that in certain contents pose a risk to humans, animals and the environment around us. It recently became a serious problem worldwide. Poor air quality may damage human health and also affect the structure and the function of ecosystems (EEA 2014) because as the pollutants enter the atmosphere, the coarse and heavy particles could rapidly be deposited near the source of the emission and the other, indeed fine particles could be transported to the long distances following the movement towards the atmospheric masses. Air pollution shows acute and chronic effects on human health by causing several health problems and affecting different systems and organs such as respiratory irritation, heart disease, lung cancer, asthmatic attacks, premature mortality and reduced life expectancy (Kibria 2016; EEA 2013). The atmosphere is one of the most important routes of the distribution of pollutants in the environment. Metals are environmental pollutants, and their toxicity is a problem of increasing the harmful in terms of ecological, food and environmental issues (Nagajyoti et al. 2010). They are naturally present in the earth's crust and naturally exist in the environment as various chemicals. Under different conditions and anthropogenic releasing, they may enter the environment as contaminants. The metals may be emitted into the environment through the various natural and man-made processes such as agricultural and industrial manufacturing, traffic and mining operations (Lee and von Lehmden 1973). These processes depend on a number of factors such as (i) the physical and chemical characteristics of the pollutants (e.g. particle size and their chemical form), (ii) the source characteristics of the emission (e.g. height of the chimney, composition of the pollutant mixture) and (iii) environmental factors (e.g. winds and precipitation) (Treshow 1984).

After the emission and the atmospheric transport in different distances, the trace elements leave the atmosphere by wet and dry deposition and accumulate on the earth's surface. Metals are very persistent in the environment. The transport of metals to different aquatic, air, soil and biota ecosystems makes them able to be assimilated by the humans through inhalation, drinking and feeding. Cu and Zn are essential elements for humans and plants, and As, Cd, Cr, Pb and Hg are classified as elements of high toxicity that pose a significant risk to public health even at a low concentration level of exposure. The toxicity level depends on several factors, i.e. the chemical species, the dose, the route of exposure and the age, gender, genetics and nutritional status of exposed individuals (Tchounwou et al. 2012). In general, many metals are essential for humans and plants when they occur in normal content, but may turn harmfully in high content and may become toxic that may pose negative consequences to humans and plants. Trace metals were defined as hazardous chemicals because they are toxic, non-degradable and bioaccumulative (Mann et al. 2011). Due to the solid form (excluding Hg) of metals and their compounds, they are transported into the atmosphere only as aerosol particles mostly derived from anthropogenic sources, including mineral particles, industrial processes, traffic emission, burning of different fossil fuels and waste incineration (Freedman and Hutchinson 1981; Bargagli 1998), and by natural sources such as volcanic eruptions, rock erosion, forest burning, wind-driven soil particles, marine salt crystals, etc. (Csavina et al. 2012). Cadmium (Cd), mercury (Hg), lead (Pb), arsenic (As), copper (Cu), chromium (Cr), nickel (Ni), cobalt (Co) and zinc (Zn) are the most toxic metals that may pose different risk to human health and the environment (Järup 2003; Tchounwou et al. 2012). The contamination of the air by toxic metals is among the main threats coming by atmospheric depositions, because it is a component of the air that we breathe (Qarri et al. 2019). The severity of adverse health effects is related to the type of heavy metal and its chemical form and is also time- and dose-dependent (Järup 2003; Tchounwou et al. 2012).

Air quality monitoring can be performed by measuring pollutants directly in the air or in depositions, building models that describe the distribution of pollutants or using different biomonitors. Studies on atmospheric pollution have consistently been limited by high costs of classical analytical methods, as well as the difficulties in performing extensive monitoring in time and space. Consequently, there has been a growing interest in using the methods of indirect monitoring, such as biomonitoring. Many studies have shown a very good ability of bryophyte moss to uptake atmospheric pollutants in the form of the trapped particulate matter within the surface layer or the dissolved liquids (Varela et al. 2015; Fernandez and Carballeira 2002; Tremper et al. 2004; Berg et al. 1995; Berg and Steinnes 1997). The use of mosses as biomonitors of metals' atmospheric deposition on a regional scale appeared first in Scandinavian countries more than four decades ago. This is now widely accepted as a very efficient method of estimating atmospheric heavy metal deposition. Due to technical difficulties and high cost of atmospheric pollutant monitoring, the European Air Quality Directives 2004/107/EC and 2008/50/EC only demand for the determination of few atmospheric contaminants (e.g. Pb, Cd, As, Ni, Hg and benzo[a]pyrene) (De Nicola et al. 2013).

The study of atmospheric deposits of most toxic metals Cd, Hg, Pb, As and Ni in the territory of Albania is included. Arsenic, cadmium, lead, mercury and nickel are mainly emitted from various natural and anthropogenic processes. Windblown soil dust, forest fires, volcano eruption and sea spray (Cd, Pb and Ni) are the most important natural emission sources of these elements in the atmosphere. They are released into the atmosphere from metal smelters, pesticides used in agriculture (As and Cd), traffic, mining industry, shipping activity in coastal areas, etc. The main natural sources of Hg emissions are the diffusion from the earth's mantle through the lithosphere, evaporation from the sea surface and geothermal activity (EEA 2013). Some EU countries, including Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Denmark, Greece, Hungary, Italy, Latvia, Lithuania, Luxembourg, North Macedonia, the Netherlands, Poland, Romania, Slovenia and Spain, exceed the critical loads for Hg across nearly 90% or more of their ecosystem area (EEA 2013). The differences between the pollution levels of each country are linked with the respective contribution coming from national and foreign sources. The metal content of the atmosphere is also linked with PM content in the air (Guerreiro et al. 2014). The PM2.5 emissions were reduced mostly as a consequence of installing secondary abatement technologies such as electrostatic precipitators and wet scrubbers, but the countries like Albania, Bosnia and Herzegovina, Iceland, North Macedonia, Montenegro, Norway, Serbia and Switzerland do not show a significant reduction between 1990 and 2013 (Colette et al. 2016; Guerreiro et al. 2014).

2.2 Material and Method

2.2.1 An Overview of Albania

Albania positioned in Southeast Europe and in the western part of the Balkan Peninsula. It is bordered with the Adriatic and Ionian seas in the west (Fig. 2.1) and a land border of only 720 km long. Albania is characterized by a Mediterranean climate in the west and a Mediterranean-continental climate in the east. The average annual rainfall is about 1430 mm.

The activities in ex-industrial sites of copper, chromium, iron-nickel and oil industries have produced several million tons of industrial waste impacting the surrounding environment that had adversely affected the natural resources, followed by a potential health risk for the people continuously exposed to the polluted areas (UNDP Albania 2010). The lack of a national network onto air quality monitoring as well as the data onto morbidity caused by air pollution makes it impossible to assess the health impact of air pollution in Albania (UNDP Albania 2010).

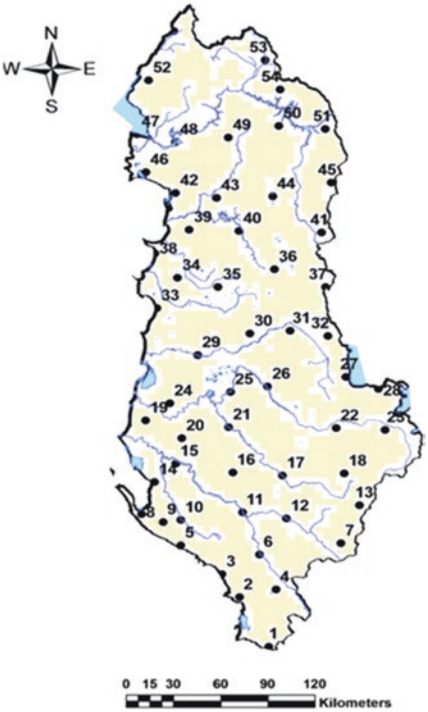

Fig. 2.1 The map of Albania and its geographical position (Centred at the latitude 41° 00′ north of the equator and the longitude 20° 00′ east of Greenwich)

2.2.2 *Sampling*

Sampling was performed during a relatively dry season of August–September 2015, according to the guidelines of the LRTAP Convention-ICP Vegetation protocol and sampling strategy of the European Programme on Biomonitoring of Heavy Metal Atmospheric Deposition (Frontasyeva and Harmens 2015). Moss samples were collected at a total of 55 sampling sites. A systematic sampling scheme was applied (EPA QA/G-5S 2002) with a homogeneous distribution of more or less equal densities (≈2 moss samples/1000 km^2). One of the recommended moss species, *H. cupressiforme*, widely spread in Albania, was used in this study as an air pollution bioindicator. The sampling locations were situated at least 300 m away from main roads or buildings and 100 m from small roads and single houses. Most of the samples were collected in open areas. Composite moss samples were formed by five to ten sub-samples collected within an area of 50 × 50 m^2. The sampling sites in mountain areas were positioned in deep valleys by keeping the altitude lower than 1000 m. Only five samples in the southeast (Korça-Pogradec region) were collected from the plateau region with an elevation lower than 1300 m. To prevent any contamination of the samples, sampling and sample handling were performed using disposable polyethylene gloves. The distribution of the sampling sites is shown in Fig. 2.1.

2.2.3 *Sample Preparation and Chemical Analysis*

Unwashed green and greenish-brown parts of moss leaves that represent 3–5 years of plant growth were cleaned from the foreign materials that adhered to the surface of the samples such as tree bark, lichens, soil dust and the dead materials. The samples were dried to constant weight for 48 h at 30–35 °C. To reduce the particle size and satisfy the conditions for homogeneity of the sample, the samples were ground and homogenized prior to the analysis using a plastic mortar and pestle.

Moss samples were totally digested using a microwave digestion system (Mars, CEM, USA) according to the method presented by Stafilov et al. (2018). The instrumental parameters of both methods are published at Stafilov et al. (2018) and Balabanova et al. (2010). The content of Co, Cu, Ni and Zn in the moss samples was determined by inductively coupled plasma atomic emission spectrometric (ICP-AES) (Varian, 715ES). As and Cd were determined by electrothermal atomic absorption spectrometry (ETAAS) (Varian, SpectrAA 640Z). ICP-AES and ETAAS analysis were performed at the Institute of Chemistry, Faculty of Science, Ss. Cyril and Methodius University, Skopje, North Macedonia.

Mercury was determined by cold vapour atomic absorption spectrometry (CVAAS) (Varian 10+ equipped with a homemade cold vapour system) as described by Lazo and Cullaj (2002). Wet digestion of homogeneous sub-sample (0.5 g sample and 10 ml nitric acid 9:1 v/v in half pressure Teflon tubes) was applied for Hg digestion as given by Lazo et al. (2018).

Three replicates per moss samples were digested, and three replicate measurements per digests were made during the analysis. The limits of the quantification (LOQ) for the elements determined with ICP-AES and CVAAS methods are shown in Table 2.1. The limits of quantification were calculated as 10 SD of the lowest instrumental measurements of the blanks (Qarri et al. 2013).

2.2.4 *Quality Control*

M2 and M3 international moss reference materials (Steinnes et al. 1997) were used to perform the quality control of the ICP-AES and ETAAS analysis (Stafilov et al. 2018). Moss reference materials were analysed together with moss samples at each ten-sample interval. The determined ICP-AES reference values reported by Stafilov et al. (2018) and Balabanova et al. (2010) were in good agreements with the recommended values. In addition, blank samples were measured in parallel to the analysis of the moss samples. International plant reference material IAEA-140/TM (*Fucus homogenate*) was used for checking the quality control of the CVAAS analysis (Lazo et al. 2018). The recovery of the investigated elements was checked by standard addition method. It ranged between 98.5% and 101.2% for ICP-AES and 96.9% and 103.2% for AAS. Differences between the mean contents of Hg from the respective certified values of reference material were within 15%. The recommended (Steinnes et al. 1997; Harmens et al. 2010) and the obtained values of element concentration of M2 and M3 reference moss samples are shown in Table 2.2. The mean contents of the elements under investigation were in good agreement with the certified data.

2.2.5 *Data Processing and Statistical Analysis*

Statistical analysis was used to investigate the variability and spatial distribution of the elements' atmospheric deposition and to assess the most probable pollution sources of the elements under investigation. Aiming to compensate the natural variability and to distinguish the anthropogenic variability of concentration data of the

Table 2.1 The limits of the quantification (LOQ) of the elements (mg kg^{1}) determined with ICP-AES and CVAAS methods

Elements	LOQ	Elements	LOQ	Elements	LOQ
As	0.035	Cr	0.12	Ni	0.85
Cd	0.02	Cu	0.035	Pb	0.85
Co	0.024	Hg	0.008	Zn	0.007

Table 2.2 The recommended values (Steinnes et al. 1997; Harmens et al. 2010) and the obtained values for element concentrations in reference moss samples M2 and M3 (ICP-AES analysis)

Element	M2 (in mg kg^{-1})		M3 (in mg kg^{-1})	
	CV ± SD	DV ± SD	CV ± SD	DV ± SD
As	0.98 ± 0.07	0.94 ± 0.03	0.105 ± 0.007	0.15 ± 0.02
Cd	0.454 ± 0.019	0.43 ± 0.03	0.106 ± 0.05	0.177 ± 0.02
Ni	16.3 ± 0.9	14.7 ± 0.61	0.95 ± 0.08	1.14 ± 0.08
Pb	6.37 ± 0.43	5.67 ± 0.52	3.33 ± 0.25	3.63 ± 0.39
Cr	0.97 ± 0.17	0.96 ± 0.06	0.67 ± 0.19	0.59 ± 0.02
Cu	68.7 ± 2.5	59.2 ± 2.83	3.76 ± 0.23	4.58 ± 0.14
Zn	36.1 ± 1.2	32.5 ± 0.92	25.4 ± 1.1	26.2 ± 0.78

CV certified value, *DV* determined value

elements, the data were normalized by using Li as normaliser element (Loring and Rantala 1992). The method was also used by Bekteshi et al. (2015) to 2010 moss survey concentration data of Albania.

The relationship between the elements in moss was tested by Spearman correlation analysis, confirmed by the statistical significance level, $P < 0.01$. Factor analysis (FA) was applied as an extension of the correlation analysis to assess the relationship between elements present in moss samples and to identify the most important factors that probably affect the association of the elements in the same factor. FA may explore the hidden multivariate structures of the data (Astel et al. 2008; Reimann et al. 2002) and may clarify the link between the elements that tend to have similar origins or to subsequently develop similar associations on the data matrix. Each factor was explained on the basis of the associations of the elements extracted from the correlation matrix. The statistical analysis was performed using the Minitab 17 software package. ArcGIS 10.2 in combination with local deterministic methods and the inverse distance weighting were used for mapping the spatial distribution of the elements.

2.2.6 *Health Risk Assessment*

The concentration data of the most toxic elements (As, Cd, Hg and Pb) are used to calculate the chronic daily intake dose (CDI) of vegetables intake by using the formula given by EPA regulations (EPA 1989, 2001):

$$\mathrm{CDI}_{\mathrm{ing}} = \frac{C * \mathrm{IR} * \mathrm{ED} * \mathrm{EF}}{\mathrm{BW} * \mathrm{AT}} * 10^{-6} \qquad (2.1)$$

where:

$\mathrm{CDI}_{\mathrm{ing}}$ – chronic daily intake dose caused by metal content in edible vegetables (100 mg)

C – metal contents in specific sites (mg kg^{-1}) (in mosses in this case)

IR – vegetable ingestion rate (100 g/day)
ED – exposure duration (24 year)
EF – exposure frequency (day/a) (365 day year^{-1})
BW – average body weight (70 kg for adults)
AT_{nc} – average exposure time (365 × 24 day)
AT_{c} – average exposure time (365 × 70 day)
LT – lifetime expected (70 year)
10^{-6} – conversion factor (kg-mg)

The probability of cancer (c) risk as a result of exposure to the carcinogenic hazards was calculated according to Eq. (2.1) (EPA 2001). The carcinogenic risk (CR) was estimated by the sum of the non-cancer (nc) and cancer (c) risk of each carcinogenic element (As, Cd, Hg and Pb). The $CR < 1 \times 10^{-6}$ is considered as a carcinogenic target risk, and if the $CR > 1 \times 10^{-4}$, it is qualified as an unacceptable value (Candeias et al. 2014).

2.3 Result and Discussion

2.3.1 Trace Metal Concentrations in Moss Samples

Only the most toxic elements that show negative health impact and environmental risk are included. The most important parameters, such as mean, median, minimum, maximum, coefficient of variation (CV%), skewness and kurtosis, are shown in Table 2.3.

The sequence of the distribution of elements in moss samples was Hg < Cd < As < Pb < Ni. All the elements (except Hg) show high variations (CV% > 75%) indicating that the data are affected by different factors among different areas. It is followed by high values of skewness and kurtosis indicating a high asymmetry of the concentration data, wide spreading of the data and strong geographical variations of the elements in moss samples. High metal concentrations in different locations may be associated with geochemical factors and local anthropogenic sources or

Table 2.3 Descriptive statistical analysis of 2015 AMS trace metal data ($N = 55$)

Element	Mean	Median	GeoMean	StDev	CV%	Min	Max	Q1	Q3	Sk	K
Hg	0.059	0.049	0.049	0.040	68	0.011	0.213	0.033	0.068	1.86	4.20
Cd	0.222	0.116	0.150	0.233	105	0.045	1.015	0.083	0.247	1.98	3.32
As	0.621	0.418	0.478	0.565	91	0.130	3.182	0.318	0.734	2.71	8.58
Pb	3.011	2.376	2.06	2.80	93	0.510	14.54	1.162	3.921	2.08	5.46
Ni	17.05	7.57	8.89	23.5	138	0.680	108	4.220	19.02	2.56	6.54
Cr	10.65	9.27	8.01	9.69	91	2.21	66	4.57	13.34	3.65	19.5
Co	1.323	1.051	1.12	0.855	65	0.354	4.239	0.757	1.648	1.77	3.27
Zn	23.28	18.41	20.54	16.21	70	10.28	108	16.19	22.50	3.48	14.1

Sk skewness, *K* kurtosis

long-range transport of the pollutants. The disparity on the distribution of the concentration data indicates that the data are affected by several factors. The concentration data onto Hg are characterized by moderate variation and look likely more stable.

2.3.2 Spatial Distribution of As, Cd, Cr, Co, Cu, Hg, Pb, Ni and Zn

The study of As, Cd, Hg, Pb and Ni in moss samples of this study followed the European Air Quality Directives 2004/107/EC and 2008/50/EC that only demand these elements as the most toxic (de Nicola et al. 2013). As the emissions of heavy metals were considerably decreased in the Europe (Harmens et al. 2015) and EMEP region (Ilyin et al. 2013) over the last two decades, there are still evident the exceedances of critical loads for Hg across nearly 90% or more of ecosystem area of Balkan area, Denmark, Italy, Latvia, Lithuania, Luxembourg, the Netherlands, Poland and Spain (EEA 2013). Beside the exposure to the high ambient air concentrations, higher than the limit or target values of ambient air concentrations above the limit or target values is a local problem in a few areas in Europe (Harmens et al. 2015, EEA 2013). The content of metals in the air is strongly linked with the content of PM. The PM2.5 emissions were reduced mostly in Europe as a consequence of improving technologies, but the Balkan countries, like Albania, Bosnia and Herzegovina, Montenegro, North Macedonia and Serbia, as well as Iceland, Norway and Switzerland did not show a significant reduction between 1990 and 2013 (Colette et al. 2016). The spatial distribution and the factors affecting the emission of As, Cd, Hg, Pb and Ni in Albania are discussed. The elements As, Cd, Hg, Pb and Ni are released into the atmosphere from both natural and anthropogenic sources. The main anthropogenic sources include metal smelters and fuel combustion, traffic, waste incineration, windblown soil dust and mining industry. The main natural sources of Hg emissions are diffusion from the earth's mantle through the lithosphere, evaporation from the sea surface and geothermal activity (EEA 2013).

2.3.2.1 Arsenic

Arsenic concentrations show high differences in moss samples (CV% = 91% > 75%). The concentration data were followed by high values of skewness and kurtosis (2.7 and 8.6, respectively) indicating a high disparity in its distribution pattern throughout the country. It is probably derived from the local emission sources and long-range atmospheric transport. As content in moss samples ranged from 0.13 to 3.18 mg kg^{-1} with an average content of 0.62 mg kg^{-1}, higher than the As concentration range founded in 2010 AMS (0.5–2.86 mg kg^{-1} and the average content of 0.48 mg kg^{-1}). To distinguish the contributions from the natural and anthropogenic sources, the concentration data of As are normalized by using Li as a typical

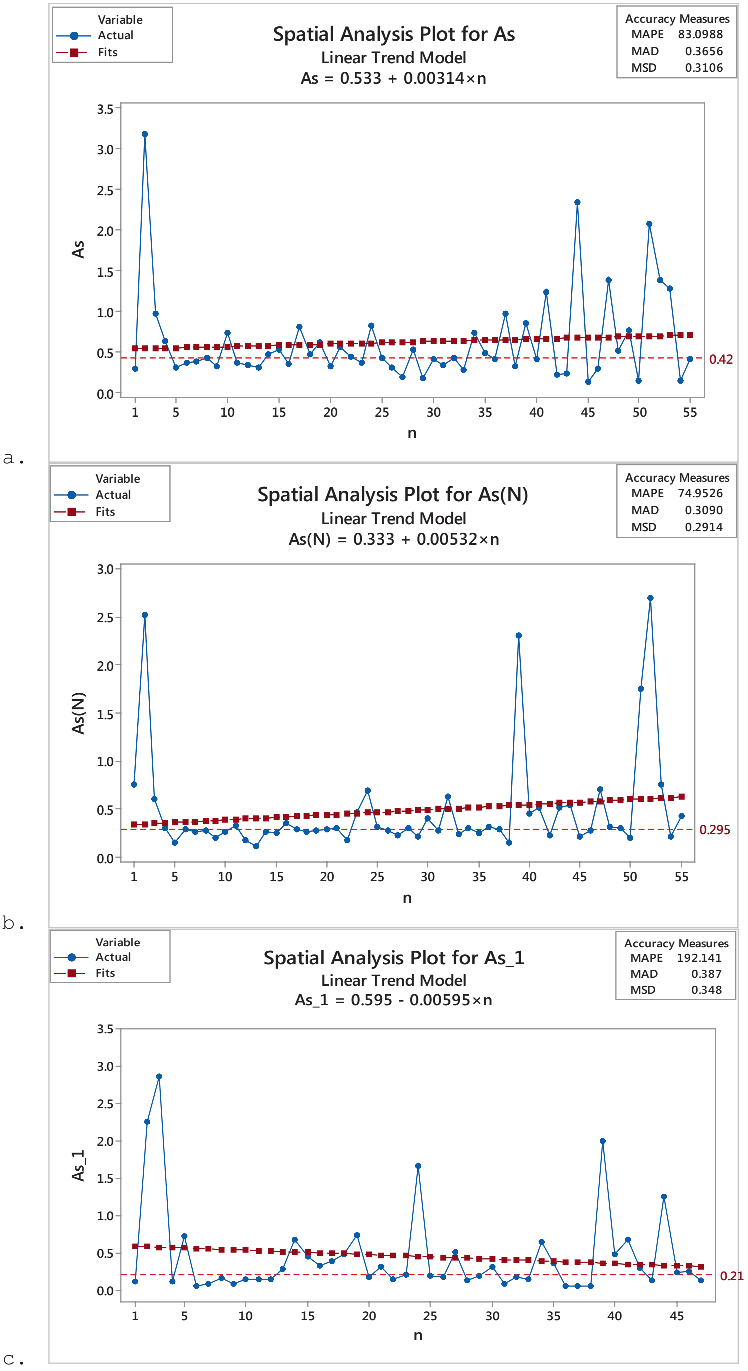

Fig. 2.2 The spatial analysis plot (linear model) of As. (**a**) 2015 AMS data, (**b**) 2010 AMS data and (**c**) 2015 AMS Li normalized data

lithogenic element (Loring and Rantala 1992). The distributions of concentration data and Li normalized data of As are shown in Fig. 2.2. The distribution of 2010 concentration data of As is shown in the same Fig. 2.2.

The spatial analysis of As (linear model) shows local enrichment of As mainly positioned in the southern and northern parts of the country. The As anomalies are probably linked with the combination of the long-range transport and strong effects of local emission sources such as geogenic factor and windblown dust from industrial and mining waste deposits in the north and the use of pesticides in the south particularly in the past decades. As normalized data show high As content in fewer stations than was found by spatial distribution of concentration data. It was mostly evident in agriculture areas (St. 2 and St. 3 in the south) that were probably derived from the use of pesticides in agriculture activity. The next high As contents were found in St. 39, St. 51 and St. 52 (Lozhan, Puke, Fushë-Arrëz) that are probably derived from geogenic factors, mining activity and the windblown mineral fine particles from the mineral waste deposits. The median concentration of As_{2015} is higher than the median concentration of As_{2010}. The differences founded in long-term pollution level of metals depend on a number of factors including changes of anthropogenic and secondary emission data, long-term climatic trends, changes of land use, etc. (Colette et al. 2016).

Moderate and significant correlations were found between As and the normalized As (As(N)) data ($r > 0.6$, $p < 0.001$), indicating the stronger anthropogenic effect to As concentration in 2015 moss samples. It shows weak and significant correlation with Cd ($r = 0.372$, $p = 0.005$). Arsenic did not correlate with Hg, a

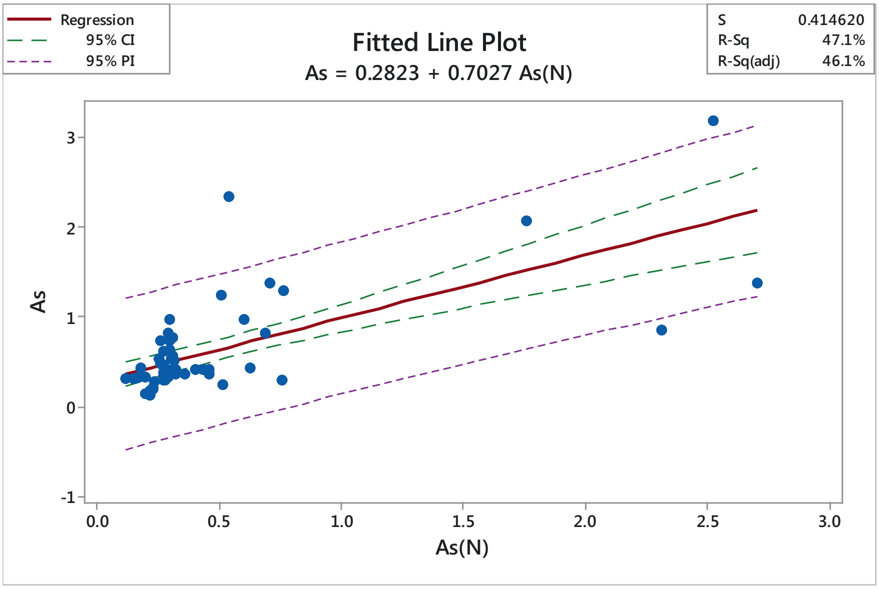

Fig. 2.3 Linear regression analysis of As vs. As(N)

typical element of long-range atmospheric transport, indicating that it is mostly affected by the local emission sources and less by the long-range transport. The mutual correlation of As-As(N) is verified by the linear regression analysis (Fig. 2.3).

2.3.2.2 Cadmium

Cd content in moss samples ranged between 0.045 mg kg^{-1} and 1.015 mg kg^{-1} with an average value of 0.222 mg kg^{-1} and a median of 0.116 mg kg^{-1}. It shows very high variation (CV% = 105% > 75%). The difference between the first quartile, Q1, and the minimum values (0.038 mg kg^{-1}) is lower than the difference between the third quartile, Q3, and the maximum value (0.738 mg kg^{-1}) indicating that the concentration data are skewed right. The distribution pattern of Cd in 2015 mosses looks likely different compared with the distribution pattern of Cd in 2010 mosses. Some new anomalies are evident on 2015 spatial distribution plot which are positioned in the new sampling sites added in the north part of the country on 2015 moss survey (in total 55 sampling sites on 2015 and 47 sampling sites on 2010 moss survey). The differences observed among different areas (Fig. 2.4) indicate the effects of different local factors and/or climatic changes between two moss biomonitoring surveys. By comparing 2015 moss concentration data with 2015 moss normalized data, it is clearly showed that the anthropogenic effects are higher in the areas of mining industry (St. 42, 43 and 52, Librazhd, Scanderbeg and Fushë-Arrëz). The sites with high Cd content (Fig. 2.4a and b) made it possible to distinguish the dual effects of natural and anthropogenic sources. The major sources for Cd emission to the air from natural sources are volcanoes, airborne soil particles, sea spray, biogenic material and forest fires. The weathering of rocks is a main natural Cd source that releases cadmium to soils and aquatic systems (www.who.int) and in air mostly as windblown dust particles Nordic Council of Ministers (2003).

The linear spatial analysis of Cd showed a higher level of Cd content in 2015 moss samples (n; n is the number of sampling sites from 1 to 55) compared to the Cd content in 2010 moss samples (Cd = 0.111 + 0.00144 × n). Cd content shows a higher increase at 2015 mosses compared to the 2010 mosses from the south to the north direction of the country. The highest content was found in NE-SE metal mineralization belt of Cr, Cu, Ni and Fe deposits indicating the effects of geogenic and mining local industry sources. The same with As, differences founded between Cd content on 2015 and 2010 moss samples are probably affected by the changes in long-range transport of pollution, the changes in climatic trends, changes of land use, etc. (Colette et al. 2016).

Cadmium showed a very strong and significant correlation with the normalized concentration Cd ($r = 0.755$, $p = 0.000$) probably indicating that the effect of anthropogenic sources is stronger than the natural emission sources. It shows weak and significant correlation ($r = 0.33$, $p < 0.05$) with Ni. The elemental contents which are captured by moss samples can be part of the emissions within particulate matter from different sources that create a very complex mixture in the air, and sometimes

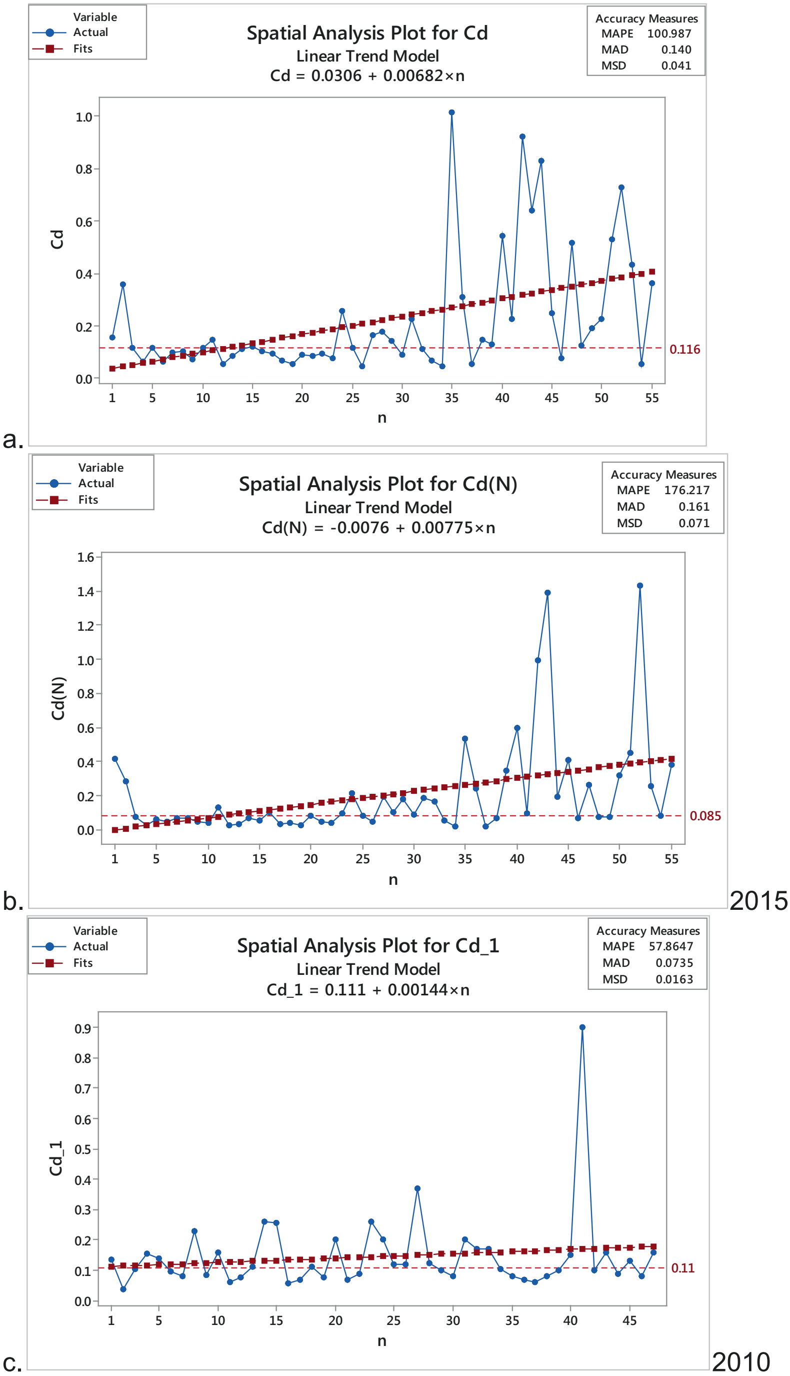

Fig. 2.4 Spatial analysis plot of Cd (linear model). (**a**) 2015 Cd concentration data; (**b**) 2015 Cd normalized concentration data; (**c**) 2010 Cd concentration data

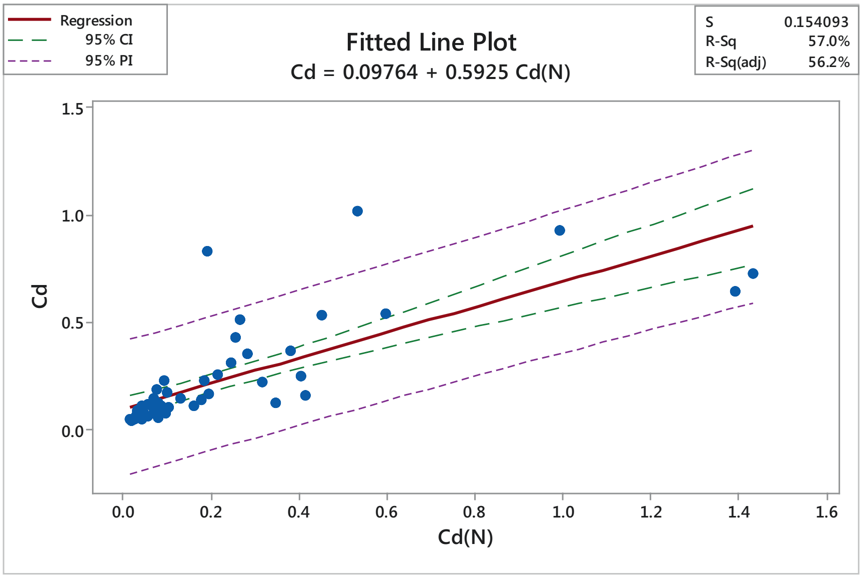

Fig. 2.5 Linear regression analysis of Cd vs. Cd(N)

the associations between elements are not easy to be explained. The correlation of Cd vs. Cd(N) is verified by the linear regression analysis (Fig. 2.5).

2.3.2.3 Chromium

Chromium (Cr) is a naturally occurring element present in the earth's crust. It enters into the environment from natural and anthropogenic sources. The largest contributions of Cr release are metal processing, mining, metallurgical, refractory and chemical industries. It is well known that the Cr hazard is linked with the exposure level and the oxidation state of Cr, with the highest toxicity of the Cr^{6+}. Cr content in moss samples ranged from 2.21 to 66.2 mg kg^{-1} with an average content of 10.65 mg kg^{-1}, lower than the respective 2010 AMS concentration range (1.47–262 mg kg^{-1} (INAA analysis) and an average concentration of 27 mg kg^{-1}). Similar to the 2010 concentration data of 2010, the 2015 Cr concentrations in moss samples show high differences and high coefficient of variation (CV% = 91% > 75%). It is followed by high values of skewness and kurtosis (3.65 and 19.5, respectively) indicating a high disparity in its distribution pattern throughout the country that mostly reflects high effects of the local emission sources in the areas with high Cr content, as well as the long-range atmospheric transport in the rest part. To distinguish the contributions from the natural and anthropogenic sources, the concentration data are normalized by using Li as a typical lithogenic element (Loring and Rantala 1992). The distributions of concentration data and Li normalized data are

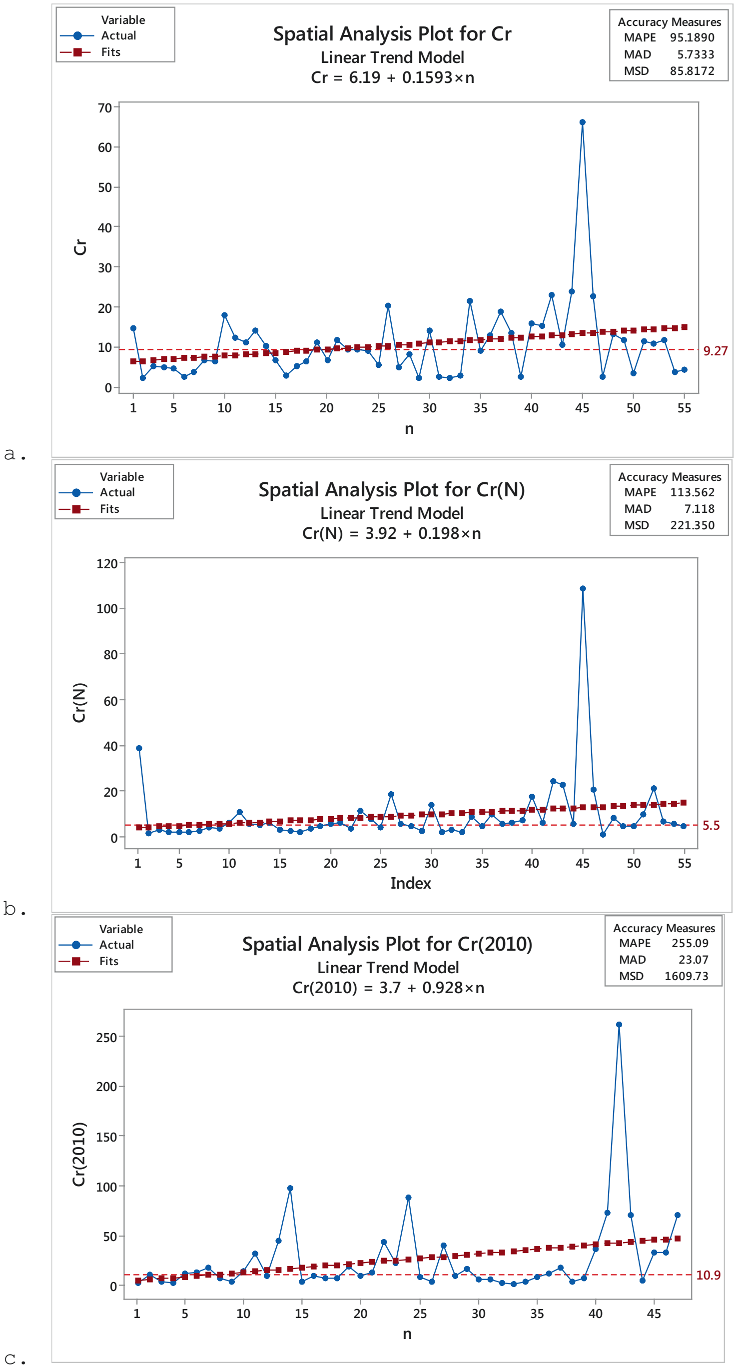

Fig. 2.6 The spatial analysis plot (linear model) of Cr. (**a**) 2015 AMS data, (**b**) 2015 AMS Li normalized data and (**c**) 2010 AMS data

shown in Fig. 2.6. The distribution of 2010 concentration data of Cr is shown in Fig. 2.6c.

The spatial analysis of Cr (linear model) shows local enrichment in the North. The Cr distribution shows an increase from the south to the North ($Cr = 6.19 + 0.16n$, $n = 1–55$). The highest Cr content (66 mg kg^{-1}, DW) was found in the north, St. 44, Bulqiza site (Fig. 2.6a), that is known as the most Cr-enriched area with intensive Cr mining industry. Other anomalies, higher than the average content of Cr in moss samples (10.65 mg kg^{-1}, DW), are distributed in other regions indicating the Cr enrichment in moss samples as the result of local factors. The Cr anomalies are probably linked with the strong effects of local emission sources such as geogenic factor and windblown dust from industrial and mining waste deposits in the north and the emission from metallurgic combine of Elbasani area, iron metallurgy and ferro-chromium industry. The most important Cr anomalies linked with anthropogenic factors were evident by the spatial analysis plot of Cr normalized data (Fig. 2.6b). Relatively high Cr contents were found in fewer stations than was found by spatial distribution of concentration data. The strongest anomaly was found again in Bulqiza area indicating high anthropogenic effects of mining industry, geological factors and windblown dust particles from mineral area and Cr waste deposits. Other anomalies are strongly linked with geogenic factors and the emissions from metallurgy of Elbasan. The median concentration of Cr_{2015} is lower than the median concentration of Cr_{2010}. The difference is probably linked with the method of the analysis of moss samples, respectively, by ICP-AES and neutron activation (ENAA) methods that use different conditions of sample preparation. ICP-AES is based on the measurements in the acid-digested samples, and ENAA measures the total amount of the elements directly in the sample without digestion. The differences are most significant in St. 44 (Bulqiza area) that is rich in Cr_2O_3 minerals that is probably not totally digested by the microwave acid digestion method.

Chromium shows strong and significant correlations with Ni and Co ($r = 0.684$ and 0.621, respectively, $p = 0.000$) indicating similar associations with these elements, known as geochemical association. Very strong and significant correlation was found between Cr and Cr(N), $r = 0.86$, $p = 0.000$, indicating the strong anthropogenic origin of Cr in moss samples. Strong correlations were found between Cr and Ni(N) and Co(N) and Pb(N) that are derived from similar anthropogenic origin of these elements in moss samples. The mutual correlations between Cr-Cr(N), Cr-Ni, Cr-Co and Cr-Pb are verified by the linear regression analysis (Fig. 2.7).

2.3.2.4 Copper

Copper (Cu) is a crustal element mostly concentrated in the complex sulphides, mafic and intermediate rocks. The concentration of Cu in soil ranges from 14 to 109 mg kg^{-1} (reference). As a component in several proteins indispensable for life (Araya et al. 2007), Cu is an essential micronutrient that occurs naturally in all plants and animals at low level of concentration, but turns toxic at high concentration level (Taylor et al. 2020, Araya et al. 2007, ATSDR 2004). Before the 1990s of

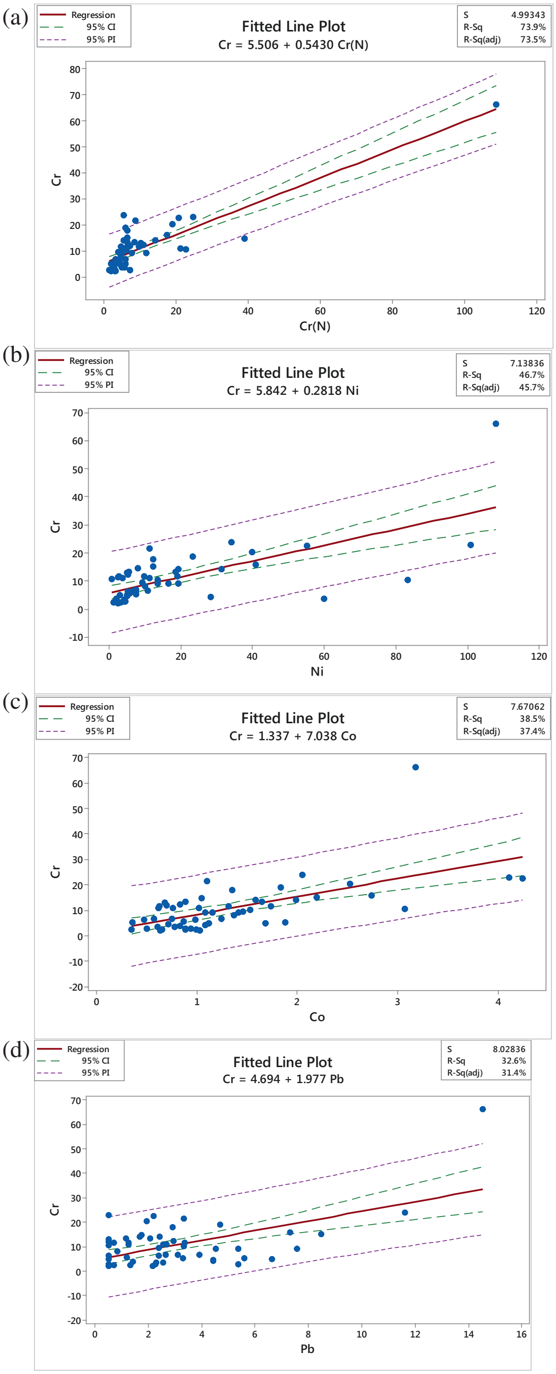

Fig. 2.7 Linear regression graphs. (**a**) Cr vs. Cr(N), (**b**) Cr vs. Ni, (**c**) Cr vs. Co, (**d**) Cr vs. Pb

the last century, Cu is extensively explored, mined and processed in the north of Albania. Due to the old technology used at that time, several tons of industrial wastes and mineral dumps are deposited in the abounded copper mines, smelters, refineries and industrial processing and waste disposal sites in different parts of the country that are considered as the main emission sources of atmospheric Cu in Albania (Lazo et al. 2019). The burning of fossil fuels and wastes, wood production and the production of the phosphate fertilizer are considered as Cu anthropogenic emission sources (Tchounwou et al. 2012). The main Cu natural sources are volcanoes, windblown dust from native soils naturally enriched with Cu, forest fires and sea spray (ATSDR 2004).

The content of Cu in moss samples showed moderate variation (CV% = 34%) and a low range of the concentration (6.1–22.4 mg kg^{-1}). The mean (10.83 mg kg^{-1}) and the median (10.0 mg kg^{-1}) concentrations are very close to each other, indicating that the data may follow the normal distribution. Low value of kurtosis ($1.19 < 3$) followed by Q3 smaller than the maximum concentration value of Cu and close to the median value indicate the data have a central tendency. The concentration data were followed by relatively low values of skewness and kurtosis (1.18 and 1.19, respectively) indicating a low disparity of the concentration data throughout the country that is probably derived from long-range atmospheric transport. To distinguish the contributions from the natural and anthropogenic sources, the concentration data of Cu are normalized by using Li as a typical lithogenic element (Loring and Rantala 1992). The distributions of concentration data and Li normalized data of As are shown in Fig. 2.8b. The distribution of 2010 concentration data of As is shown in the same Fig. 2.8c.

The spatial analysis of Cu concentration data (linear model) looks likely stable ($Cu = 10.51 + 0.0117 \times n$, $n = 1–55$) indicating the long-range transport of Cu. Small local enrichments were mainly positioned in the Cu-enriched areas in the S-E and in the north parts of the country indicating the geogenic effects derived from windblown soil dust particles in moss samples. The Cu anomalies are probably linked with the combination of the long-range transport and the effects of local emission sources such as geogenic factor and windblown dust from industrial and mining waste deposits in the north. The spatial analysis of Cu normalized concentration data (linear model) looks likely different from Cu concentration data; the spatial distribution of normalized Cu concentration data shows fewer strong anomalies than the concentration data. A strong anomaly was found in St. 1 (Konispol) and in the North, St. 39 and 41, indicating the strong anthropogenic origin of Cu in these areas probably derived from shipping activity and transboundary pollution (St. 1). The median concentration of Cu_{2015} (10.0 mg kg^{-1}) is about two times higher than the median concentration of Cu_{2010} (5.62 mg kg^{-1}). These differences are probably dependent on a number of factors including changes of anthropogenic and secondary emission data, long-term climatic trends, changes of land use, etc. (Colette et al. 2016).

Pearson correlation analysis shows strong and significant correlations between Cu and Zn ($r = 0.698$, $p = 0.000$) indicating similar origin of these elements. The Cu(N) data show strong and significant correlations with ($r > 0.6$, $p = 0.000$) with

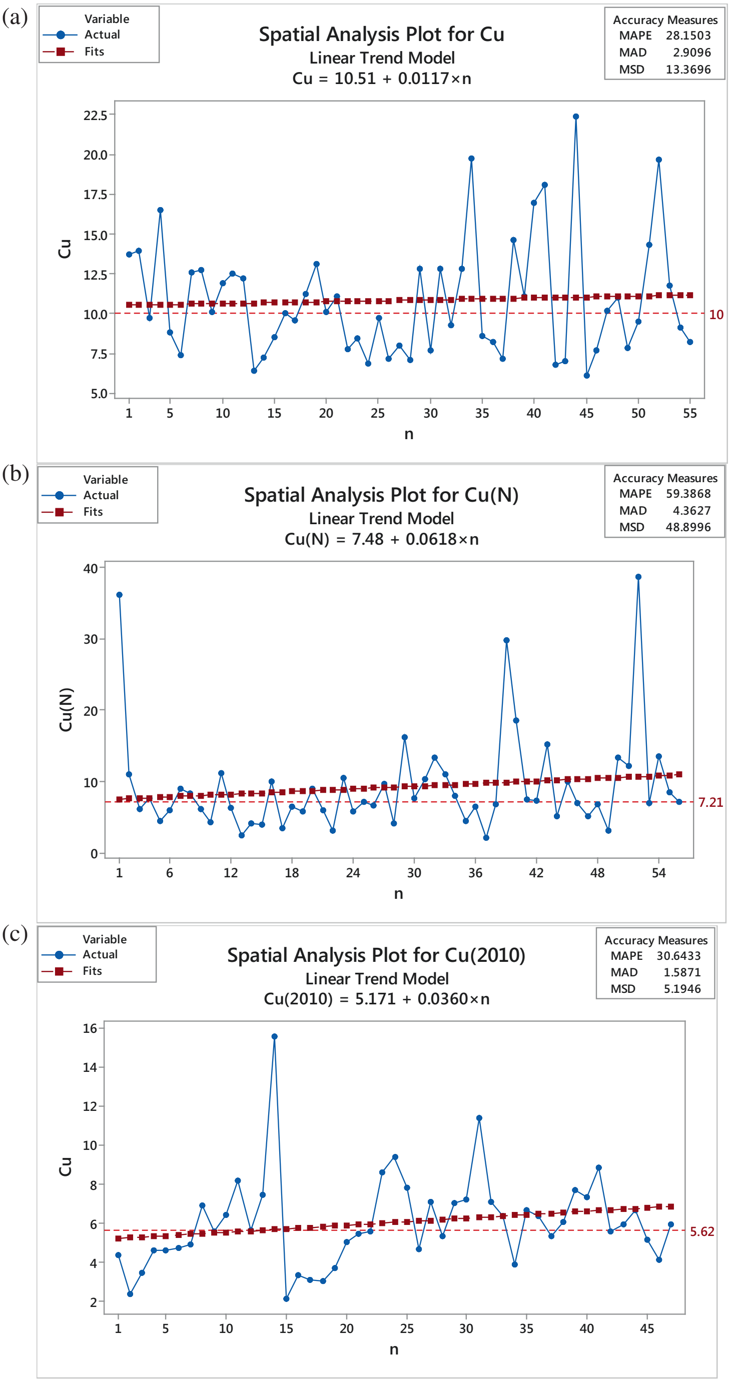

Fig. 2.8 Spatial analysis plot of Cu (linear model). (**a**) 2015 Cu concentration data; (**b**) 2015 Cu normalized concentration data; (**c**) 2010 Cu concentration data

Zn(N), Hg(N), As(N) and Cd(N) ($r = 0.580$, $p = 0.000$) indicating stronger anthropogenic effects than the natural one of these elements in 2015 moss samples. High correlation with Hg and Cd, typical elements of long-range atmospheric transport, is probably indicating the strong effect of long-range transport. The mutual high correlation between Cu and Zn is verified by linear regression analysis (Fig. 2.9). It is probably derived from the emission from the brake wear which is likely an important emission source of these elements, and the lack of correlations with Pb indicates a low source from the vehicle exhaust.

2.3.2.5 Lead

Pb is not a common element of earth crust and is mostly present in different ores and minerals (UNEP 2013). It can enter in the air through the natural and the anthropogenic processes. Pb contents in moss samples varied from 0.51 mg kg^{-1} to 14.54 mg kg^{-1}, lower than 2010 (1.34–19.7 mg kg^{-1}), while the average concentrations of 2015 and 2010 are very close to each other (3.011 mg kg^{-1} and 3.12 mg kg^{-1}, respectively). The Pb concentration in moss samples of Albania is lower compared to the mosses of the most European countries (Lazo et al. 2018; Harmens et al. 2013).

The spatial analysis plot shows diverse distribution of Pb content in the country. It is confirmed by high variation (CV% = 93% > 75%) and high values of skewness and kurtosis (2.1 and 5.5, respectively) indicating a high disparity of the Pb concentration in moss samples. The highest concentration level was found in St. 44 and 45

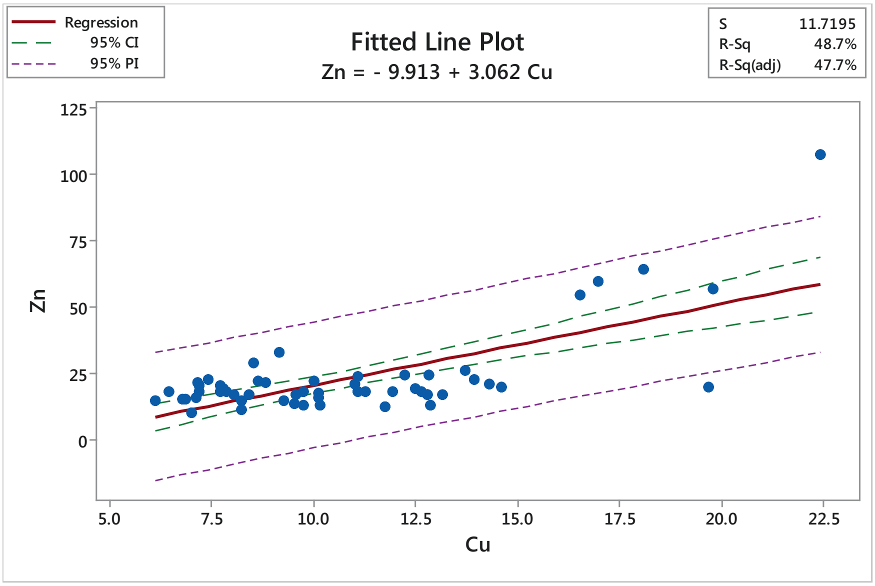

Fig. 2.9 Linear regression analysis between Cu and Zn

(Librazhd and Burrel areas). Other Pb anomalies though lower in concentration levels were found in different parts of the country indicating the different sources of Pb content in moss samples. The spatial analysis plot of the Pb normalized concentration data (Fig. 2.10b) looks likely uniform throughout the whole territory indicating low anthropogenic sources. The highest anthropogenic effects were found in St. 45 (Burrel) followed by St. 40 and 41 (Bilisht and Pogradec). Small Pb anomalies were found in St. 23 and 24 (Berat and Kuçova). All these areas are known for their ore deposits and mining activity. By comparing the spatial distribution of Pb concentration data and normalized concentration data, it is clearly showed that most of the country is affected by natural emission sources of Pb mostly caused by soil dust emission. The spatial analysis plot of Pb (linear model) shows more stable and homogenous distribution of Pb throughout the country ($Pb = 2.308 + 0.0335 \times n$; n represents the number of sampling site) (Fig. 2.10) that is probably derived from long-range transport of pollutants and local natural emission sources. The outlier points with high Pb content are probably indicating the local anthropogenic inputs of Pb. Differences observed on spatial distribution of Pb during two moss monitoring surveys probably are derived from the changes in the bioavailability of the elements caused by the climatic changes that favour dry deposition on 2010 moss survey and wet deposition on 2010 (Lazo et al. 2019, Qarri et al. 2019).

Pearson correlation analysis showed very strong and significant correlation between Pb concentration data and Pb normalized concentration (Pb(N) data ($r = 0.719$, $p = 0.000$) probably indicating that the effect of anthropogenic sources is stronger than the natural emission sources. Pb normalized concentration data show moderate and significant correlations with Ni and Ni(N) data ($r = 0.528$ and 484, $p < 0.001$, respectively). The correlation of Pb vs. Pb(N) is verified by the linear regression analysis (Fig. 2.11).

2.3.2.6 Mercury

Mercury is a global pollutant that occurs in the environment in different forms such as metallic, inorganic and organic mercury. It is a metal naturally occurring in earth's crust and is widely used in different processes and man-made products (ATSDR 1999). Hg ranged from 0.04 to 0.213 mg kg^{-1} with an average content of 0.059 mg kg^{-1}. The concentration data of Hg show a moderate variation (CV% = 68%) followed by relatively high positive values of skewness and kurtosis (1.86 and 4.2, respectively). Relatively high Hg content was found in the southern coastal area from Borsh to Ilias (St. 3 to St. 5), in the S-E (St. 35, 37–39) in the border line between Albania and Greece and in the north part of the country (St. 49). The spatial analysis plot of Hg normalized concentration data that reflect the anthropogenic Hg contribution showed a different view regarding the distribution of the Hg(N) data. The first anomaly in the S-W is still evident (St.1 to St. 5). The second area with relatively high Hg(N) values was found in the S-E (St. 38 to St. 42), and the next one zone is recorded in the north (St. 9, 51, 52 and 54).

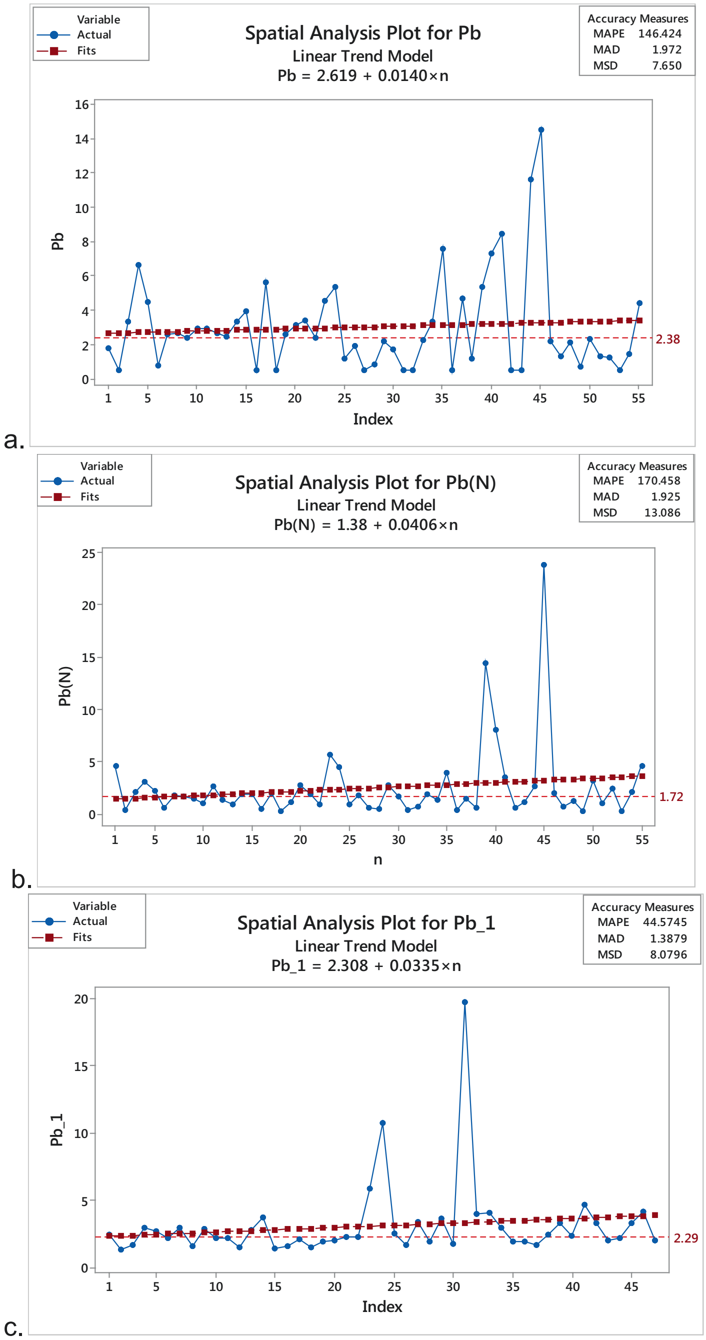

Fig. 2.10 Spatial analysis plot of Pb (linear model). (**a**) 2015 Pb concentration data; (**b**) 2015 Pb normalized concentration data; (**c**) 2010 Pb concentration data

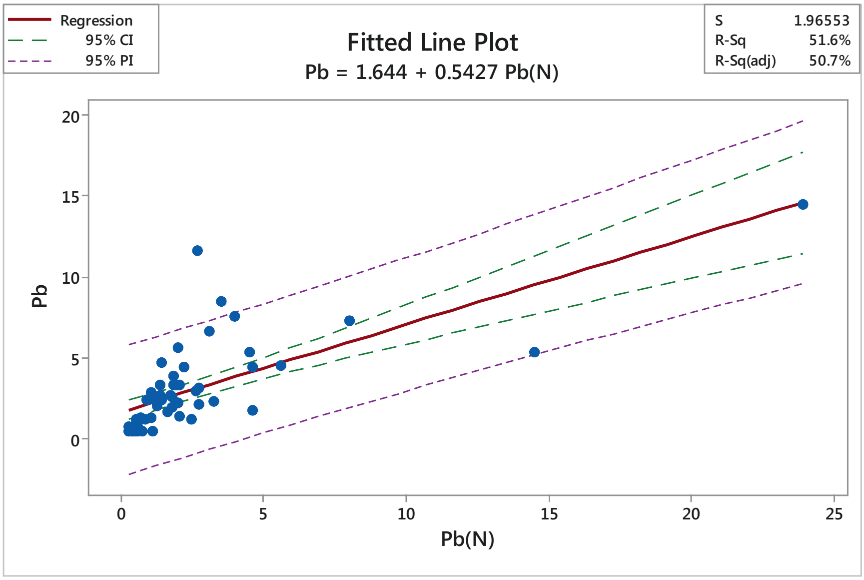

Fig. 2.11 Linear regression analysis of Cd vs. Cd(N)

The spatial distribution plots of 2010 and 2015 moss survey of Hg concentration data resulted totally different not only in distribution patterns but also in concentration levels. The anthropogenic emission from iron and steel metallurgy and waste burning was evident from two outlier points with extremely high Hg content, positioned at St. 24, in Elbasan region, and St. 29, in Kruja region (Fig. 2.12c). In general, the total Hg predominately depend on the relatively stable global/hemispheric background concentration, and only the influence from major sources may have been resulted in higher values (Wangberg et al. 2001). Beside these two points, the rest of the moss samples showed lower Hg content but still higher than that of 2015. The median concentration of Hg in 2010 moss samples (0.14 mg kg^{-1}) is about three times higher than the median concentration of Hg in 2015 mosses (0.049 mg kg^{-1}). Mercury mostly exist in gaseous forms in the atmosphere, and the climatic changes that favour Hg during dry weather of 2010 and not during wet weather of 2015 (Lazo et al. 2019, Qarri et al. 2019) could be a major factor of these differences. Thus, the long-range transport of the pollutants can be considered as the main source of Hg atmospheric deposition in Albania. Long-range transport has been pointed as an important source of mercury in Europe (Harmens et al. 2015). The anthropogenic factors such as iron and steel metallurgy, geogenic factors, transboundary pollution, crude oil and gas industry and shipping activity in the coastal areas may result in local anomalies of Hg.

Pearson correlation analysis showed strong and significant correlation between the Hg concentration data and Hg normalized concentration (Hg(N) data ($r = 0.7$, $p = 0.000$) probably indicating the effect of anthropogenic sources. Hg(N) show

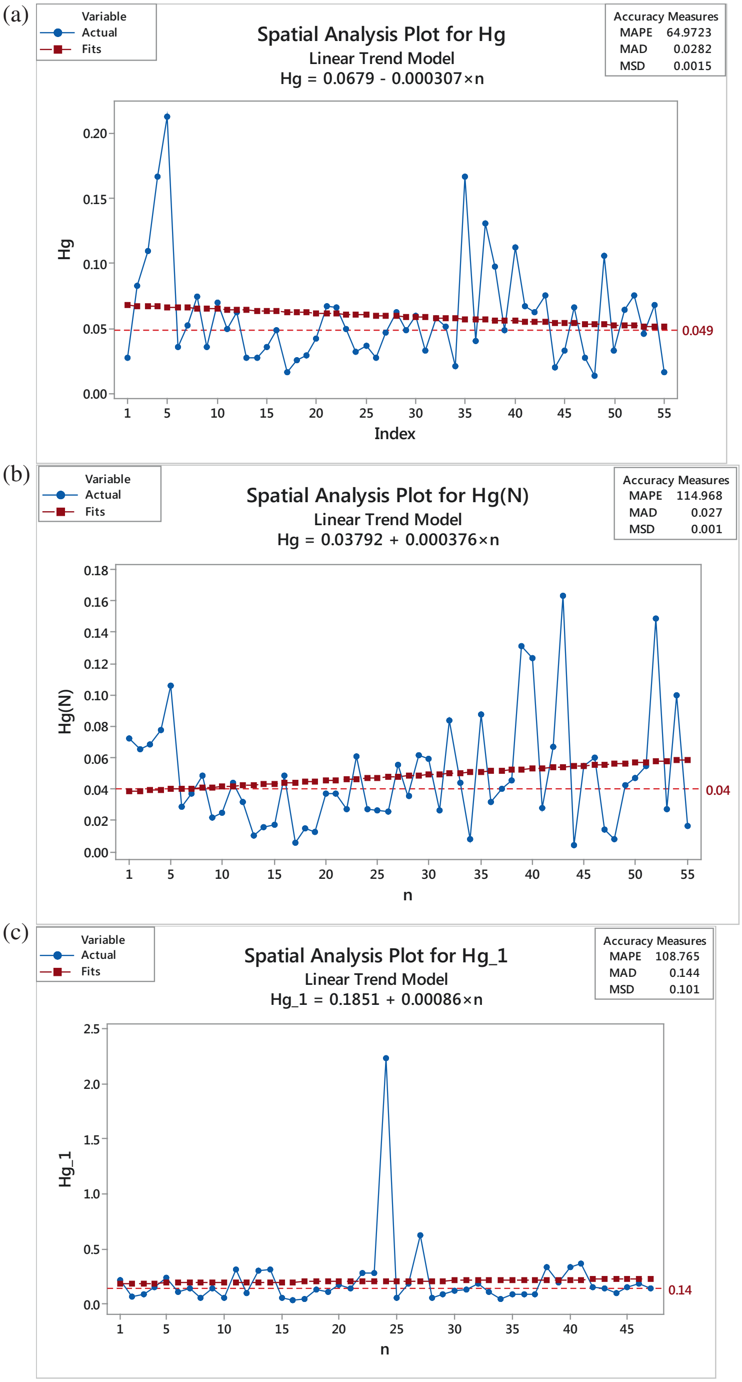

Fig. 2.12 Spatial analysis plot for Hg. (**a**) 2015 Hg concentration data; (**b**) 2015 Hg normalized concentration data; (**c**) 2010 Hg concentration data

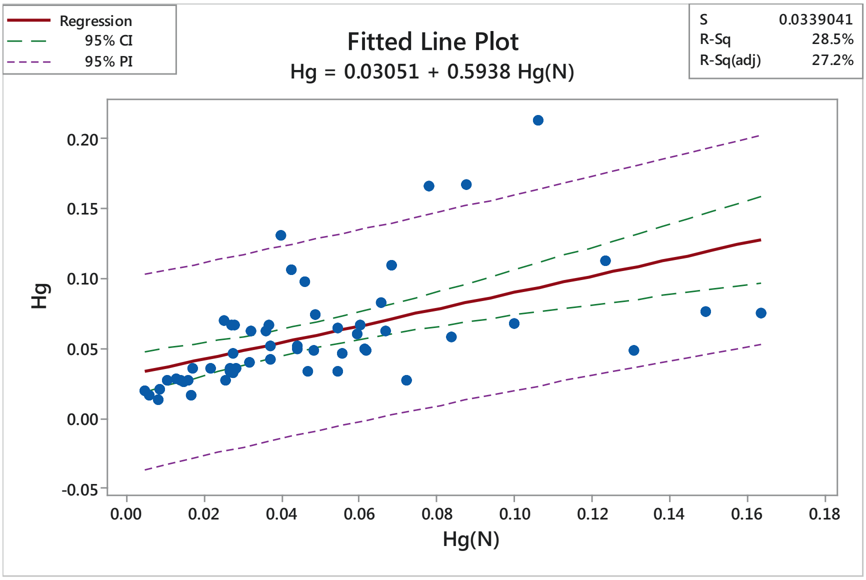

Fig. 2.13 Linear regression analysis of Hg vs. Hg(N)

moderate and significant correlation with Cd(N), As(N) and Ni(N) indicating similar anthropogenic sources. The correlation of Hg vs. Hg(N) is verified by the linear regression analysis (Fig. 2.13).

2.3.2.7 Nickel

Nickel occurs naturally in the earth's crust. It is widely distributed in the environment, in the air, water, soil and biosphere. Ni enters in the air mostly as windblown fine particles, from both natural and anthropogenic sources such as the emission from high-temperature metal processing, mining industry, fuel combustion, traffic emission and shipping activity in the coastal areas, oil and gas industry, waste incineration, cement production, etc. The chemical forms of Ni atmospheric compounds in atmosphere depend on their sources of origin. Shipping emissions contribute significantly to air pollution in coastal zone and ports (Zhang et al. 2014). Natural Ni emission sources in the atmosphere include windblown dust particles, volcanoes, vegetation, sea salt spray and forest fires (Pacyna and Pacyna, 2001). Ni is an essential micronutrient of plants that affects the field production, and it turns toxic in high concentration level, and in growing medium of the plants, it may exert adverse effects (Harasim and Filipek, 2015). On the other hand, Ni and its compounds are considered as potential human carcinogens, and atmospheric nickel is one of the major routes for human exposure (Tian et al. 2012).

Nickel contents in moss samples ranged from 0.68 mg kg^{-1} to 108 mg kg^{-1}, with an average concentration of 17.1 mg kg^{-1} and a median of 0.57 mg kg^{-1}. It shows a high variation (*CV% = 138%*) followed by high positive values of skewness and kurtosis (2.56 and 6.54, respectively) that indicate high disparity of the concentration data with a tendency of skewed right. Significant spatial variations are identified among different areas indicating the different levels of the Ni air pollution. The spatial analysis of Ni concentration data (linear model) shows a high increase from the south to the north direction of the country ($Ni = 1.4 + 0.559 \times n$; n represents the number sampling sites). By comparing the Ni spatial distributions obtained from the spatial distributions of concentration data and the normalized Ni data, it was shown that the positions of the anomalies of Ni concentration and normalized data lie in the same positions (Fig. 2.14). It is clearly indicating that the anthropogenic emission sources are predominant in Ni content in moss samples.

The anomalies with high Ni concentrations were found in the same sampling sites as Cr normalized concentrations (St. 26, 30, 39, 40, 42–46, 54 and 55), positioned in the central (St. 22 and 24) and in the eastern parts of the country. The highest Ni content was also found in Cr deposit areas (St. 42–46 and St. 54, 55). High Ni contents in moss samples of these stations are strongly linked with anthropogenic sources of windblown dust particles sourced from the areas of chromite and nickel silicate deposits and nickel-ferrous ores (Milushi 2015) indicating the high effects of geogenic factors. Moss sample of St. 26 is also affected by Ni anthropogenic emission from metallurgical combine of Elbasani area, and St. 26 is affected by Ni anthropogenic emission from the oil production industry of Kuçova area.

These findings showed that the distribution of Ni with relatively high contents is mainly localized in the areas of Ni and Cr ores and mining industry which are highly polluted by Ni sourced by windblown fine mineral dust particles. It indicates Ni content in moss is probably derived mostly from local inputs of air pollution from industry, mining activity and geogenic factors and long-range atmospheric transport. High median value of Ni in moss samples of Albania is higher than the median value of European moss survey (Harmens et al. 2015).

Similar distribution patterns were observed from spatial analysis (linear model) of 2015 and 2010 Ni concentration data that are characterized by high increase of Ni content in S-N direction ($Ni = 1.4 + 0.559 \times n$ and $Ni = -1.61 + 0.618 \times n$, respectively). The changes observed in the spatial distribution plots of 2015 and 2010 were obtained as a result of a larger number of monitoring stations in 2015 ($N = 55$ sampling sites) compared with 2010 ($N = 47$ sampling sites). Substantial changes have been identified in the Ni concentration levels of both monitoring periods. The content of Ni in the moss samples of 2015 was higher than those of 2010. This is evidenced by changes in median values, 7568 mg kg^{-1} and 5889 mg kg^{-1}, respectively. The Ni anomalies of 2015 and 2010 biomonitoring periods resulted in the same monitoring areas. The same with other elements under investigation, the differences observed on spatial distribution of Ni during 2015 and 2010 moss monitoring surveys are probably linked with the changes in the bioavailability of the

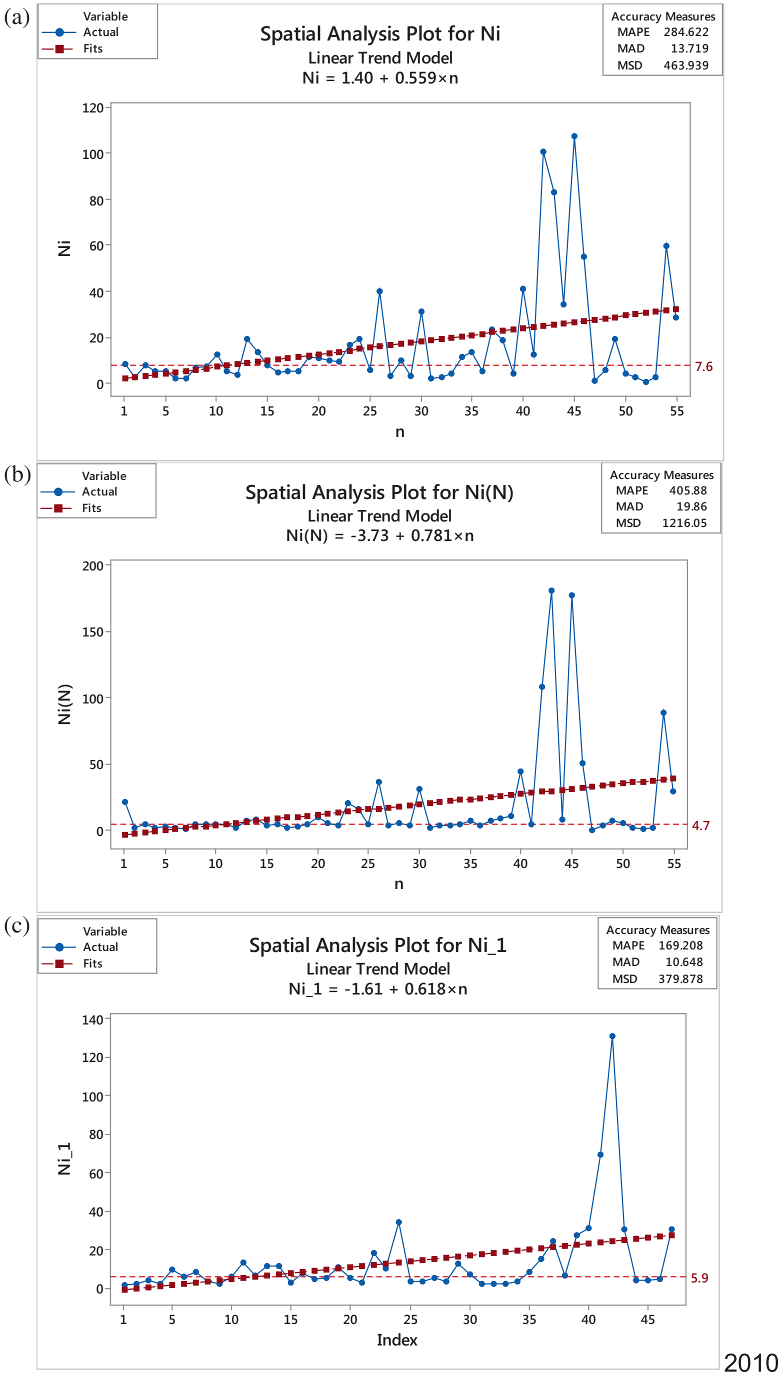

Fig. 2.14 Spatial analysis plot of Ni (linear model). (**a**) 2015 Ni concentration data; (**b**) 2015 Ni normalized concentration data; (**c**) 2010 Ni concentration data

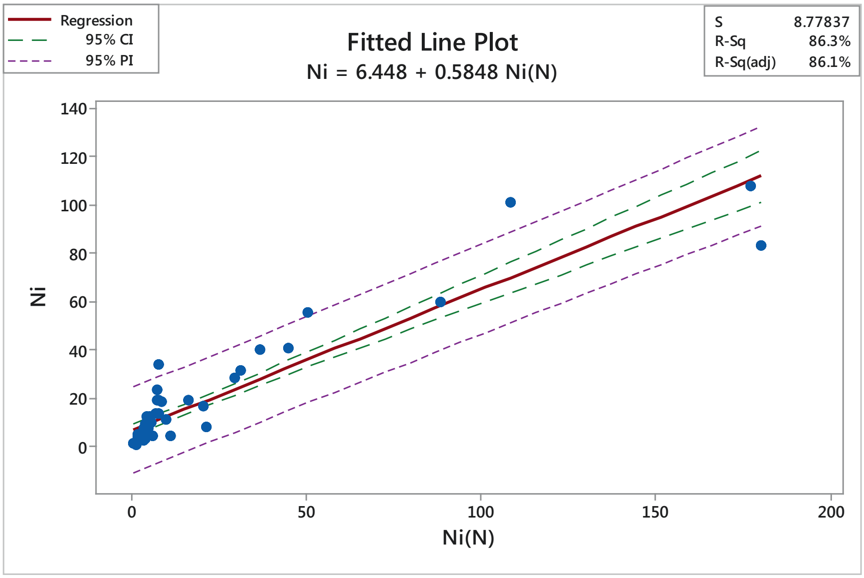

Fig. 2.15 Linear regression analysis of Ni vs. Ni(N)

elements caused by the climatic changes that favoured dry deposition on 2010 moss survey and wet deposition on 2015 (Lazo et al. 2019, Qarri et al. 2019).

Ni shows high and significant correlation ($r = 0.929$, $p = 0.000$) with Ni normalized concentration data (Ni(N)) indicating the higher input of Ni from anthropogenic sources than from natural one. The correlation of Ni vs. Ni(N) is verified by the linear regression analysis (Fig. 2.15). The geochemical associations of Ni with the mafic minerals (UNEP 2013) and ultramafic rocks that contain appreciable content of Ni (SGS 2005) which are present in the N-E and the eastern parts of Albania (Milushi 2015) indicate the effects of geogenic factors to Ni content derived from the soil dust fine particles and mining industry of these area. Ni is also in sulphide deposits that contain low Ni content. At last, the soil geochemistry, mining activity, emission from ferro-chromium metallurgy, traffic emission and oil industry may explain high Ni levels recorded at specific areas.

2.3.2.8 Cobalt

Cobalt is a naturally occurring element that has similar chemical and geochemical properties as iron and nickel (ATSDR 2004). It is dispersed in the environment at low concentration. As other metals, Co enters the environment through natural and anthropogenic sources. It is naturally released into the atmosphere by windblown soil dust, sea spray, volcanic eruptions and forest fires and from anthropogenic sources such as ore deposits, ore smelting facilities, air and highway traffic or other industrial pollution (ATSDR 2004). Cobalt released into the atmosphere in particulate form that is associated with particulate matter is principally dispersed as wind-blown soil dust (Kim et al. 2006). Co is an essential element that plays a beneficial role in plant growth and development (Arif et al. 2016). The biochemical cobalt compound, vitamin B12 (cyanocobalamin), plays an important role for good health of humans and animals (Leyssens et al. 2017). The health effects of Co are characterized by complex clinical syndromes, mainly as neurological (e.g. hearing and visual impairment), cardiovascular and endocrine deficits (Leyssens et al. 2017; Kim et al. 2006).

The content of Co in moss samples varied from 0.354 mg kg^{-1} to 4.24 mg kg^{-1}. The average (1.323 mg kg^{-1}) or median (1.051 mg kg^{-1}) concentrations of Co in moss are higher than in terrestrial plants (0.48 mg kg^{-1}, cited in Kim et al. 2006 from Bowen 1966). Co shows a moderate variation (*CV% = 65%*) followed by high positive values of skewness (1.77) and a kurtosis of 3.27 indicating a moderate disparity of the concentration data with a tendency of following the log-normal distribution. The spatial analysis of Co concentration data (linear model) shows (Fig. 2.16) a significant spatial variation among the areas with a visible increment from the south and the north indicating the different levels of the Co air pollution in these areas (Co = *0.898 + 0.0152 × n, where n represents the number sampling sites*). The anomalies with high Co concentration data and the normalized data are positioned in the same sampling sites, indicating that the anthropogenic emission sources are predominant in Co content in moss samples. The anomalies with high Co concentrations were found in the same sampling sites as Ni indicating similar origin of Co and Ni in moss samples. It dialled a strong and significant correlation between Co and Ni ($r = 0.807$, $p = 0.000$), Ni normalized concentration data (Ni(N), $r = 0.654$, $p = 0.000$), and Cr and Cr(N) ($r = 0.621$, $p = 0.000$, and $r = 0.488$, $p = 0.000$, respectively) indicating similar natural and anthropogenic sources with these elements. Co(N) shows a strong and significant correlation with Co data (Co, $r = 0.765$, $p = 0.000$), with Cr(N), Cd(N) and Pb(N) ($r = 0.699$, $p = 0.000$; $r = 0.604$, $p = 0.000$; and $r = 0.699$, $p = 0.000$, respectively) indicating similar anthropogenic sources with these elements. High correlation between Co(N) and Ni(N) is tested by linear regression line (Fig. 2.17).

2.3.2.9 Zinc

Zinc occurs naturally in soil with the content in the ranges from 10 to 300 mg kg^{-1}. It is a chalcophile metallic element and is present in the sphalerites (ZnS) founded in the north of Albania (Milushi 2015). Zn enters in the environment from natural and anthropogenic emission sources, such as mining, steel processing, coal combustion and waste incineration. Zn compounds are used extensively as anti-oxidants and as detergent/dispersant improving agents for motor oil (Tchounwou et al. 2012) and are present in the vehicle brakes and tire wear emission sources and in motor vehicle exhaust. In the global scale, emission of Zn is similar in magnitude, and the

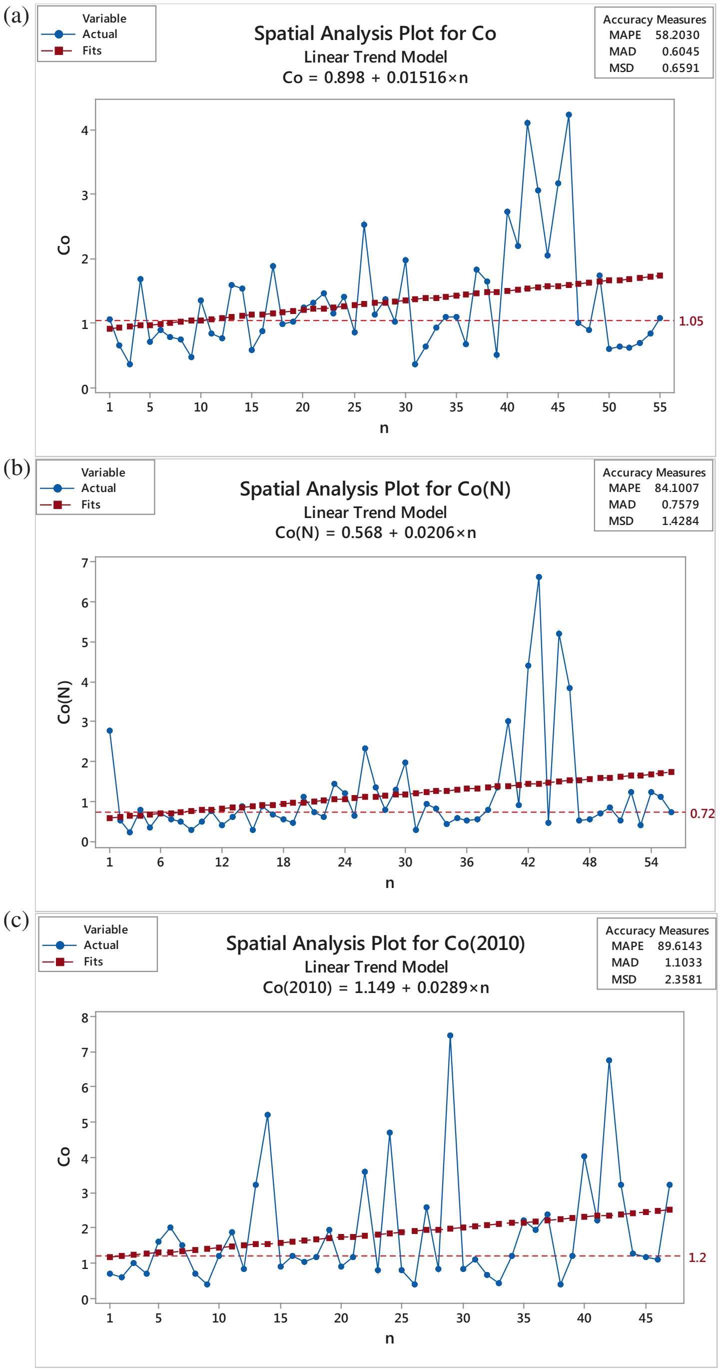

Fig. 2.16 Spatial analysis plot of Co (linear model). (**a**) 2015 Co concentration data; (**b**) 2015 Co normalized concentration data; (**c**) 2010 Co concentration data

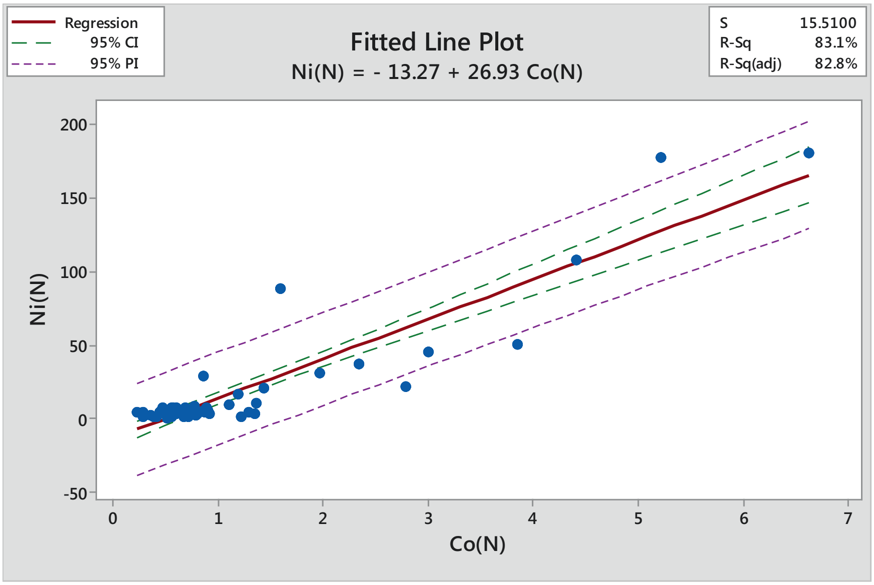

Fig. 2.17 Linear regression analysis of Co vs. Co(N)

differences in the local scale from anthropogenic sources may dominate (Tchounwou et al. 2012).

Zn is an essential element that is required for biochemical and physiological functions, but in excess amount and/or at very low content may provide a variety of adverse effects and human diseases (Tchounwou et al. 2012). Zinc deficiency or excessively high levels may enhance susceptibility to carcinogenesis (Choudhury et al. 2005). One organ where zinc is prominently involved in cell death is the brain, and cytotoxicity in consequence of ischemia or trauma involves the accumulation of free zinc (Plum et al. 2010).

Zn content in moss samples varied in a wide range, from 10.3 mg kg^{-1} to 108 mg kg^{-1}, with an average concentration of 23.3 mg kg^{-1} and a median of 18.4 mg kg^{-1} indicating a higher population of concentration data at low values. It is confirmed also by the value of the coefficient of the variation (CV% = 70%, moderate variation) and the values of the first and the third quartiles, Q1 = 16.2 mg kg^{-1} and Q3 = 22.5 mg kg^{-1}, close to the median value of the concentration data. The spatial analysis of the Zn concentration data (linear model) (Fig. 2.18) shows an increase in the north ($Zn = 21.1 + 0.078 \times n$, $n = 1–55$). The spatial analysis of concentration data and normalized data shows higher Zn content in relatively higher traffic areas indicating that the anthropogenic emission from traffic emission has a local effect in Zn distribution in moss samples. Besides, the long-range transport is an important source of Zn in Albania. By comparing the median concentrations of Zn in 2015 (18.4 mg kg^{-1}) and 2010 (13.9 mg kg^{-1}) moss samples, and the equations of the respective linear models ($Zn_{2015} = 21.1 + 0.078 \times n$, $n = 1–5$, and

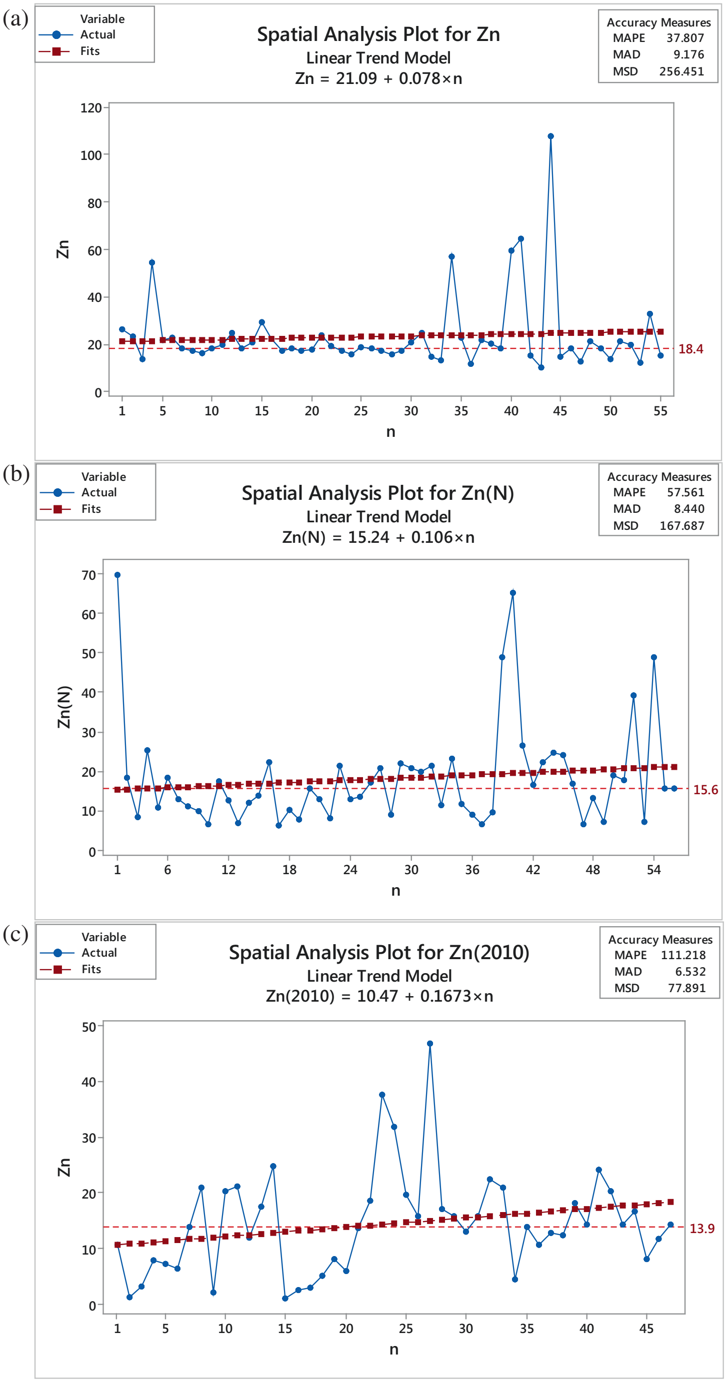

Fig. 2.18 Spatial analysis plot of Zn (linear model). (**a**) 2015 Zn concentration data; (**b**) 2015 Zn normalized concentration data; (**c**) 2010 Zn concentration data

$Zn_{2010} = 10.5 + 0.167 \times n$, $n = 1–47$), it is clear that the Zn content in 2015 moss samples is higher than those of 2010.

Zn concentration data show high and significant correlations with Cu ($r = 0.7$, $p = 0.000$) and Pb ($r = 0.62$, $p = 0.000$) and moderate and significant correlation with Cd ($r = 0.41$, $p = 0.002$), typical elements of traffic emission. The normalized Zn(N) data show high and significant correlations with Cu(N) ($r = 0.80$, $p = 0.000$) and moderate and significant correlation with Hg(N) $r = 0.58$, $p = 0.000$) and (Pb(N) ($r = 0.42$, $p = 0.002$) derived from similar anthropogenic sources from long-range transport of the pollutants.

2.3.3 Evaluation of the Health Risk

Humans and the environment are directly in contact with the pollutants through the atmospheric deposition. It makes it possible to evaluate the exposure level of humans and/or ecosystems. The manner in which the data are summarized depends upon the site characteristic and the pathways being evaluated. According to EPA (EPA 1989), the exposure assessment quantifies the magnitude, frequency and duration of exposure for the populations and exposure pathways selected for quantitative evaluation through the estimation of the exposure concentrations in the selected pathway-specific intakes. After the atmospheric deposition of pollutants, they could enter the ecosystem (soil, biota and water) and be in dermal contact with the humans or take it by food chain and inhalation. Moss biomonitoring of atmospheric deposition involves for chemical analysis of metals the green and/or green-brown parts of the moss which represent the 3–5 years of moss growth period. It makes it possible to survey the spatial and temporal trends of deposition in integral way that should be considered as a long-term exposure, and the exposure concentrations should be representative as long-term averages. The levels of metal contents in moss samples are similar to those in vegetables in Albania (Lazo et al. 2003; Kasa et al. 2015) and in other global regions (Ali and Al-Qahtani 2012; Gupta et al. 2013; Taghipour and Mosaferi 2013; McBride et al. 2014; Souri et al. 2019) and in edible wild plants (nettle leaves) (Nica et al. 2012). Very high contents of Cd, Co, Cr, Cu, Ni, Pb and Zn are reported in plants (58 plant spp. in total) collected at serpentine sites in Albania (Shallari et al. 1998).

The sequence of the medians of the carcinogenic elements was Hg < Cd < As <Co < Pb. 73% of the total area ($N = 40$ moss samples) resulting in CR values higher than the target risk ($1 \times 10^{-6} < CR < 1 \times 10^{-5}$) and lower than the CR unacceptable value of 1×10^{-4} (Fig. 2.19). Allajbeu et al. (2017) had discussed the pollution level based on different factors and indexes such as the contamination factor of the elements, the pollution load indexes, and the risk index values, and have pointed out the areas with high content of toxic metals as the areas with high risk to humans. The highest CR values were found in the areas of mining industry such as Bulqiza (St. 40), Burrel (St. 36) and Alarup and Memëlisht (St. 27 and 28).

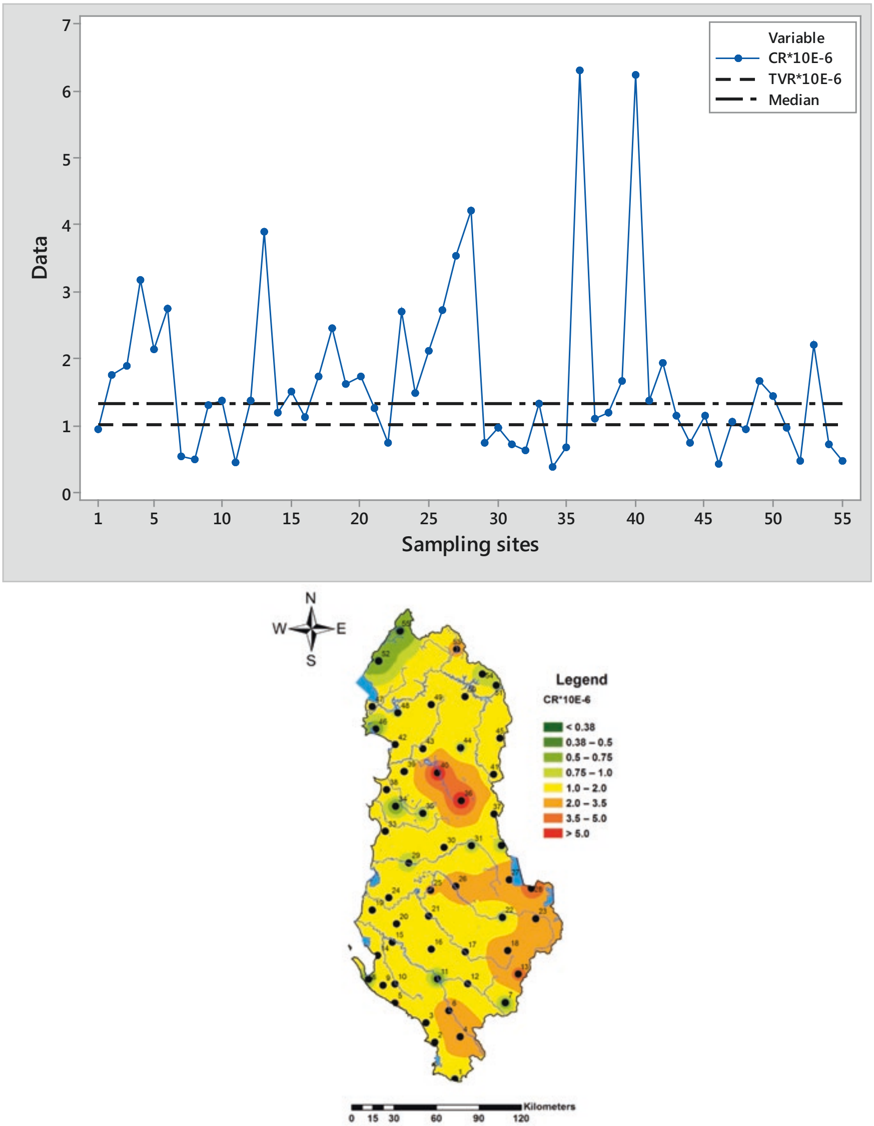

Fig. 2.19 Distribution of CR values among the sampling sites

Based on the age-standardized death rate (per 100,000 population) calculated from the data given by the World Life Expectancy (https://www.

Table 2.4 Age-standardized cancer death rate (per 100,000 population)

Country	Albania	Austria	Greece	Sweden	Italy	North Macedonia
Rate	111.08	126.21	115.51	121.71	123.79	139.72

worldlifeexpectancy.com/world-health-rankings), Albania stands among the neighbouring countries (Greece and North Macedonia) and other European countries, Sweden, Italy and Austria (Table 2.4). From the same source, the highest death rate is from lung cancer (rate, 27.64; world rank, 21), stomach cancer (rate, 18.46; world rank, 4) and breast cancer (rate, 17.09; world rank, 88), all linked with the exposure to toxic metals (Yuan et al. 2016; Carver and Gallicchio 2018; Pamphlett et al. 2020).

2.3.4 *Multivariate Analysis*

Factor analysis (FA) was applied onto concentration (C) and the normalized concentration (C(N)) data to assess the relationship between elements present in moss samples and to identify the most important factors probably affecting the association of the elements in the same factor. The number of the significant factors was selected for eigenvalues >1. The loadings >0.5 are under the consideration that represents relatively high and significant correlations between the elements of the same factor. The data of FA are shown in Table 2.5.

Four main factors that represented 77.7% of the total variance were extracted from FA. *The first factor* (F1) represents 27.4% of the total variance and is linked

Table 2.5 Factor analysis of the correlation matrix of concentration (C) and the normalized concentration (N) data (varimax rotation sorted rotated factor loadings and communalities)

Variable	Factor 1	Factor 2	Factor 3	Factor 4	Communality
Ni	0.944	0.000	0.000	0.000	0.930
NiN	0.911	0.000	0.000	0.000	0.907
CoN	0.894	0.000	0.000	0.000	0.928
Co	0.840	0.000	0.627	0.000	0.776
Cr	0.631	0.000	0.597	0.000	0.809
CrN	0.631	0.000	0.597	0.000	0.852
CuN	0.000	0.955	0.000	0.000	0.931
HgN	0.000	0.774	0.000	0.000	0.746
ZnN	0.000	0.746	0.000	0.000	0.733
AsN	0.000	0.725	0.000	0.000	0.654
CdN	0.531	0.681	0.000	0.000	0.868
Pb	0.000	0.000	0.865	0.000	0.884
PbN	0.000	0.000	0.813	0.000	0.883
Zn	0.000	0.000	0.000	0.769	0.815
Cu	0.000	0.000	0.000	0.739	0.796
CD	0.000	0.000	0.000	0.713	0.766
As	0.000	0.000	0.000	0.663	0.517
Hg	0.000	0.000	0.000	0.000	0.187
Variance	4.9230	3.6059	2.7628	2.6897	13.9814
% Var	0.274	0.200	0.153	0.149	0.777

with Ni, Co, Cr and their normalized data. Factor loadings of the Ni(N), Co(N) and Cr(N) are very close to the loadings of the concentration data indicating the high impact and similar anthropogenic sources of these elements. Cd(N) is also associated with F1 indicating similar anthropogenic sources of Cd with Ni, Co and Cr, probably from high-temperature metal processing industry.

The second factor (F2) represents 20% of the total variance and is linked with the normalized data of Cu, Pb, Cr, As and Cd indicating similar anthropogenic sources of these elements.

The third factor (F3) represents 15.3% of the total variance and is linked with Pb, Pb(N), Cr and Cr(N). Factor loadings of the normalized data are very close to the loadings of the concentration data indicating the high impact and similar anthropogenic sources of these elements.

The fourth factor (F4) that represents 14.9% of the total variance is associated with high loadings of Zn, Cu, Cd and As concentration data indicating similar origin of these elements.

The elements under investigation are known as typical anthropogenic elements that were clearly tested by factor analysis such as mining and Cr-Ni processing industry (F1), metal industry (F2), road transport (F3) and long-range transport of elements derived from electricity and heat production (F4).

2.4 Conclusions

Moss survey data in combination with the statistical analysis produced a detailed and up-to-date coverage of trace metals in moss samples that directly indicate the metal atmospheric deposition of Albania. Through the spatial analysis (linear model) of metal concentration data, it was possible to predict the spatial extinction of the areas with high metal concentrations where the local factors were suggested to be dominant in air pollution that pose threats from metal depositions.

The local emission sources and long-range atmospheric transport of the pollutants show significant contributions to atmospheric deposition of metals in Albania. Carcinogenic trace metal contamination is among the main threat component of atmospheric deposition since it may pose risk to the human health. Significant differences were found in the concentrations of As, Cr, Cu, Hg, Ni, Pb and Zn in moss samples. The N-E part of Albania is also characterized as a contaminated area from the elements under investigation. It may affect the crop cultivation for human or animal consumption that may accumulate trace metals and thus may pose a risk to human and animal health. The western agricultural land area could be considered as a safer area for food production. The anthropogenic sources of the elements are likely higher than their natural background.

The examination of the distribution profile, the levels of the potential anthropogenic elements that pose high risk to the human health (As, Cd, Hg and Pb) and the correlation between elements pointed out that the traffic emission, oil and gas industry, shipping activity in the coastal areas, geochemical and geological factors, long-range transport and the emission from metallurgy are the most probable sources of toxic metals in moss samples and atmospheric deposition over the territory of Albania.

The estimated carcinogenic risk (CR) linked with the carcinogenic element (As, Cd, Hg and Pb) ranged at $1 \times 10^{-6} < CR < 1 \times 10^{-5}$. The median value of CR (1.36×10^{-6}) is higher than the carcinogenic target risk value (1×10^{-6}) and lower than the Cr unacceptable value ($CR > 1 \times 10^{-4}$). The sequence of the medians of the carcinogenic elements was Hg < Cd < As < Pb. High death rates from lung cancer and stomach cancer in Albania are linked with the level of carcinogenic and toxic metals in atmospheric deposition as it is strongly linked with the air we breathe.

Factor analysis indicated the anthropogenic sources of the elements such as mining and Cr-Ni processing industry (F1), metal industry (F2), road transport (F3) and long-range transport of elements derived from electricity and heat production (F4).

Moss biomonitoring survey provides a unique opportunity for the assessment of metal contamination in atmospheric deposition in local and continental range, and it is a strong indicium for the continuous and detailed monitoring in problematic areas.

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Chapter 3
Moss Biomonitoring of Air Pollution Around the Coal Mine and Bitola Thermoelectric Power Plant, North Macedonia

Trajče Stafilov, Robert Šajn, Mila Arapčeska, and Ivan Kungulovski

Abstract From the obtained results of the air pollution study performed by moss biomonitoring in the vicinity of the coal mine and Bitola thermoelectric power plant, North Macedonia, it could be concluded that the analysed elements are grouped into four associations of elements: Factor 1 (Fe, Al, V, Ni, Li, Cr, Co, Ba, Pb, Sr, Mn and Zn), Factor 2 (Cu, Mo, Ag, Mg and Zn), Factor 3 (K, P, Na, Mg and Cu) and Factor 4 (As, Cd). The elements included in the first association are present in varying contents throughout the studied area with higher contents of these elements found in the moss samples from the central part of the area. It was found that some of the elements have higher content in the parts close to thermoelectric power plant (TEP) and in the area between TEP and the city of Bitola. This is the case for the spatial distribution of chromium, zinc and copper from Factors 2 and 3. The spatial distribution maps indicate the areas under the dust from the fly ash and slag deposit in the TEP Bitola because these elements are present in higher content in the waste materials than in the surrounding soil.

Keywords Air pollution · Moss biomonitoring · Coal mine · Thermoelectric power plant · Bitola · North Macedonia

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3.1 Introduction

The environmental pollution at hazardous levels for livings presents a global problem and as a macro-case to monitor. Some sub-disciplines occurred with time in order to consider the realistic environmental conditions. Arguably, the understanding of atmospheric pollution is one of the more emergent areas of the environmental science. Atmospheric pollution represents solutions or suspensions of minute amounts of harmful compounds in the air (Vallero 2008). The degree and extent of environmental changes over the last decades have given a new urgency and relevance for detection and understanding of environmental changes, due to human activities, which have altered global biogeochemical cycling of heavy metals and other pollutants (Hock and Seifert 2003; Agarval (2009); Blagnytė and Paliulis 2010; Glukhov and Prokhorova 2011). Monitoring toxic air pollutants is needed for understanding their spatial and temporal distribution and ultimately to minimise their harmful effects. In addition to direct physical and chemical methods of air pollution monitoring, *bioindication* has also been used to evaluate air pollution risk (Vallero 2008).

Heavy metals present only a part from a plurality of harmful compounds in air. The degree and extent of metal distribution in the air depend on the emissions' frequency (Longchurst and Brebbia 2013). However, the higher contents of certain heavy metals introduced in the air provide hazardous conditions for populations and environment at all. Air pollution with heavy metals presents a global problem, but the hot spots occur and influence on local level (Gauderman et al. 2000, 2004; Harmens et al. 2010). For that issue a double type of monitoring programme should be applied. The first one should cover the larger areas, locating the hot spots in the investigating region, and hence the small area where the local emission sources of heavy metals directly influenced on the local population and its environment.

The goal of this study is to follow eventual impact of the air pollution from the urban activities and from the thermoelectric power plant REK Bitola in the Bitola region, North Macedonia, especially fly ash deposited on the fly ash damp site in the vicinity of the plant and close to the city of Bitola, by using moss biomonitoring.

3.2 Biomonitoring of Air Pollution

Bioindicators include biological processes, species or communities used to assess the quality of the environment and how it changes over time (Fergusson and Ryan 1984; Aboal et al. 2010). Changes in the environment are often attributed to anthropogenic disturbances (e.g. pollution, land use changes) or natural stressors (e.g. drought, late spring freeze), although anthropogenic stressors form the primary focus of bioindicator research. The widespread development and application of bioindicators have occurred primarily since the 1960s (Rühling and Tyler 1968). Over the years, it was expanded the repertoire of bioindicators to assist in studying all types of environments (i.e. aquatic and terrestrial), using all major taxonomic groups. However, not all biological processes, species or communities can serve as successful bioindicators. Physical, chemical and biological factors (e.g. substrate, light, temperature, competition) vary among environments (Fernandez et al. 2007, 2012).

In common usage, the terms “biomonitoring” and “bioindication” are interchangeable. However, in the scientific community, these terms have more specific meanings (Fränzle 2006). Bioindicators qualitatively assess biotic responses to environmental stress, while biomonitors quantitatively determine a response. Hereafter, the term “bioindicator” is used as a collective term to refer to all terms relating to the detection of biotic responses to environmental stress. Within this framework, there are three main functions of bioindicators: (1) to monitor the environment (physical and/or chemical changes), (2) to monitor ecological processes and (3) to monitor biodiversity (Onianwa 2001).

Examples of environmental, ecological and biodiversity indicators can be found in many different organisms inhabiting many different environments. Lichens (a symbiosis among fungi, algae and/or cyanobacteria) and bryophytes (mosses and liverworts) are often used to assess air pollution. Bryophytes serve as effective bioindicators of air quality because they have no roots and no cuticle and acquire all their nutrients from direct exposure to the atmosphere (Wolterbeek 2002). Their high surface area to volume ratio further encourages the interception and accumulation of contaminants from the air. The numerous benefits of bioindicators have spurred legislative mandates for their use in countries’ monitoring programmes around the world. Yet bioindicators are not without their problems. Like the canaries in the coal mine, we rely upon the sensitivity of some bioindicators to function as early warning signals. In some instances, we cannot discriminate natural variability from changes due to human impacts, thus limiting the applicability of bioindicators in heterogeneous environments as Ceburnis and Valiulis report (1999). Accordingly, populations of indicator species may be influenced by factors other than the disturbance or stress (e.g. disease, parasitism, competition, predation), complicating our picture of the causal mechanisms of change. A second criticism of the use of bioindicators is that their indicator ability is scale-dependent. For example, a large vertebrate indicator (e.g. a fish) may fail to indicate the biodiversity of the local insect community. Third, bioindicator species invariably have differing habitat requirements than other species in their ecosystem. Managing an ecosystem according to the habitat requirements of a particular bioindicator may fail to protect rare species with different requirements. Finally, the overall objective of bioindicators is to use a single species, or a small group of species, to assess the quality of an environment and how it changes over time, but this can represent a gross oversimplification of a complex system.

Like all management tools, it must be conscious of its flaws. However, the limitations of bioindicators are clearly overshadowed by their benefits. Bioindicators can be employed at a range of scales, from the cellular to the ecosystem level, to evaluate the health of a particular ecosystem. Bioindicators bring together information from the biological, physical and chemical components of our world that manifest themselves as changes in individual fitness, population density, community composition and ecosystem processes (Fernandez et al. 2007). From a management perspective, bioindicators inform our actions as to what is and is not biologically sustainable. Without the moss in the tundra, the cutthroat in the mountain stream and the canary in the coal mine, we may not recognise the impact of our disturbances before it is too late to do anything to prevent them.

3.2.1 Mosses as Airborne Pollution Bioindicators

Bryophytes are green land plants which lack a vascular system and are simple both morphologically and anatomically. The growth potential in bryophytes is not as highly polarised as vascular plants. Bryophytes grow in a variety of habitats especially in moist places on soil, rocks, trunks and branches of trees and fallen log. They obtain nutrients directly from substances dissolved in ambient moisture. Some substances are probably absorbed directly from the substrate by diffusion through the cells of the gametophyte. Bryophytes are used as reliable indicators of air pollution (Fernandez et al. 2007). They are exploited as *bryometer* instrument for measuring phytotoxic air pollution. Bryophytes, independently or together with lichens, can be valuable organisms in developing an Index of Atmospheric Purity (IAP) which is based on the number, frequency coverage and resistance factor of species (Ceburnis and Valiulis 1999). This index can provide a fair picture of the long-range effects of pollution in a given area (Markert et al. 2003). There are two categories of bryophytes in response to pollution:

- Bryophytes that are very sensitive to pollution and show visible symptoms of injury even in the presence of minute quantities of pollutants. These serve as good indicators of the degree of pollution and also of the nature of pollutant.
- Bryophytes that have the capacity to absorb and retain pollutants in quantities much higher than those absorbed by other plant groups growing in the same habitat. These plants trap and prevent recycling of such pollutants in the ecosystem for different periods of time. Analysis of such plants gives a fair idea about the degree of metal pollution.

Bryophytes are able to concentrate heavy metals in large amounts, greatly surpassing the absorbing capacity of vascular plants. The gametophytes of moss can accumulate iron five to ten times more readily than the vascular plants. The concentrations of Al, Ba, Cr, Cu, Fe, Ga, Ni, Pb, Ag, Ti, V, Zn and Zr were higher in bryophytes than those in angiosperms. The bryophytes are able to concentrate rare earth elements. Elements which are rarely found in other plants were found in bryophytes (Zechmeister et al. 2003; Balabanova et al. 2010; Barandovski et al. 2008, 2012, 2013).

Metals are non-degradable and once released into the environment become an integral part of the habitat. Bryophytes are able to concentrate heavy metals in large amounts than that of vascular plants. Heavy metals are absorbed either from the atmosphere or from the substrate or from both the sources. The older tissues of the plant have higher concentrations of the metallic ions as compared to the younger portions (Fernandez et al. 2012). The ability of mosses to accumulate heavy metals depends upon the total leaf surface and the number of thin-walled parenchyma cells. The use of carpet-forming bryophytes has proved to be rapid and inexpensive method for surveying heavy metal deposition in the terrestrial ecosystem (Rühling and Tyler 1968). The concentration of airborne material decreases in the plant tissues with distance from the source of pollution, and there is a wide variation in metal accumulation from species to species and from habitat to habitat under different microclimate conditions (Žibret and Šajn 2008). Among mosses the profusely branched and ramifying pleurocarps and the densely packed acrocarps are more efficient entrappers and absorbers of metal particles than the unbranched and erect acrocarps.

Mosses have been frequently used to monitor time-integrated bulk deposition of metals as a combination of wet, cloud and dry deposition, thus eliminating some of the complications of precipitation analysis due to the heterogeneity of precipitation (Markert et al. 2003). Ectohydric mosses in particular draw negligible amounts of water and minerals from the soil and instead depend almost entirely on atmospheric inputs of nutrients (Rühling and Tyler 1968). Because mosses have a high cation exchange capacity (CEC), they act as hyper-accumulators of metals and metal complexes. The metals are bound to the tissue with minimal translocation within the plant due to a lack of vascular tissue (Rühling and Tyler 1968). This results in biological tissue that can be analysed to reveal time-integrated deposition (Zechmeister et al. 2003). Additional advantages of using mosses as heavy metal biomonitors include their stationary nature, widespread geographic distribution and low genetic variability between populations. It has been shown that there is some experimental error due to heterogeneity in morphological characteristics and microenvironments among different populations (Zechmeister et al. 2003). There is also an incomplete understanding of the degree of mineral uptake by ectohydric mosses in direct contact with substrate (Gjengedal and Steinnes 1990). Despite the accuracy and precision of precipitation analysis techniques, however, mosses offer an efficient, low-cost complement for determining metal concentrations at a large number of locations and offer analyses of biologically relevant fluxes at multiple scales.

Harmens and his European colleagues (Harmens et al. 2008, 2010) have found that mosses are reliable indicators of air pollution risks to ecosystems, because they get most of their nutrients direct from the air and rain, rather than the soil. Since 2000 the European moss survey has been conducted by a special international programme (ICP Vegetation). Moss data provides a better geographic coverage than measured deposition data and can reveal more about actual atmospheric pollution at a local level (http://icpvegetation.ceh.ac.uk/).

3.3 Materials and Methods

3.3.1 Study Area

Study area is located in southwest part of the country, in Pelagonia Valley (Fig. 3.1). Thermoelectric power plant "REK Bitola" is a facility of strategic importance and primary installation for electricity generation in the country. This power plant is major polluter of the environment in the region, and it constantly creates large amounts of waste in the form of fly ash and slag (ELEM 2007; Stafilov et al. 2018a). The biggest settlement in the study area is the city of Bitola, which is the administrative and economic centre of the region. Although Pelagonia Valley is at a distance of 155 km from the Adriatic Sea, and at about 130 km from the Aegean Sea, still the Mediterranean climate influence with a temperate-continental, continental and mountainous climate is mostly felt. In this area the north wind prevails with the average speed of 2.2 m/s, the second for its constancy is the south wind with the annual average speed of 3.7 m/s and the third is the northwest wind with the average speed of 2.4 m/s (Lazarevski 1993).

The study area includes parts of two large tectonic units: the Pelagonian massif and the Western Macedonian zone. The Pelagonian massif is separated from the Western Macedonian zone by a big reverse Pelagonian fault, which is covered by young Quaternary deposits. The Pelagonian massif is built of Precambrian metamorphic and igneous rocks. The oldest rocks in the Western Macedonian zone are Paleozoic age and consist of low metamorphic schists and granitic rocks (Stafilov and Šajn 2016, 2019).

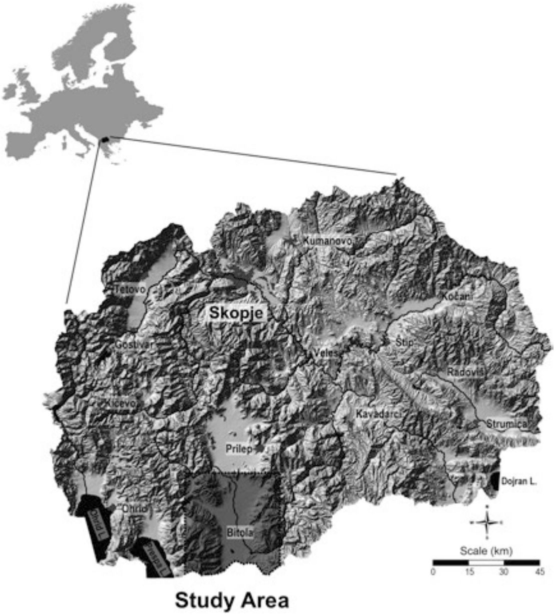


Fig. 3.1 Studying area

3.3.2 Moss Sampling

Moss sampling was done following the instructions of the monitoring manual (2015 survey): “Monitoring of atmospheric deposition of heavy metals, nitrogen and POPs in Europe using briophytes” given by the United Nations Economic Commission for Europe Convention on Long-Range Transboundary Air Pollution.

Sampling in the field was done according to the following principles:

- Each sampling point should be situated at least 3 m away from the nearest projected tree canopy: in gaps of forests (diameter >10 m) or plantations primarily (diameter >5 m), without pronounced influence from canopy drip from trees, preferably on the ground or on the surface of decaying stumps. In nitrogen-polluted regions with high density of livestock, the horizontal distances to tree crowns should be more than 7 m.
- In habitats such as open heath land, grassland or peatland, sampling below a canopy of shrubs or large-leafed herbs should be avoided, as well as areas with running water on slopes.
- Coarse contamination of moss samples (litter, soil animals) should be carefully removed. Moss cushions that are sandy and/or occupied by ants should be avoided.
- The sampling points should be located at sites representative of non-urban areas of the respective countries. In remote areas the sampling points should be at least 300 m from main roads (highways), villages and industries and at least 100 m away from smaller roads and houses.
- In order to enable comparison of the data from this survey with previous surveys, it is suggested to collect moss samples from the same (or nearby, i.e. no more than 2 km away but with the same biotope conditions) sampling points as used in the most recent moss surveys.
- It is recommended to make one composite sample from each sampling point, consisting of five to ten subsamples, if possible, collected within an area of about 50 m × 50 m.
- Only pleurocarpous mosses should be sampled. Samples should preferably be collected during the period April–October 2016. In the composite sample, only one moss species should be represented. If there is no representative moss species of Homalothecium lutescens (Hed. sw. Robins), the other species of moss could be collected: Hypnum cupressiforme (Hedw.), Lencodon scinroides and Scleropodium purum (Fig. 3.2).
- The subsamples will be placed side by side or on top of each other in suitable paper containers. The amount of fresh moss needed for metal analysis is about 1 L.
- Each locality must be given coordinates, preferably longitude and latitude (Greenwich coordinates, 360° system), suitable for common data processing.

In the composite sample, only one moss species should be represented. The subsamples should be placed side by side or on top of each other in suitable containers.

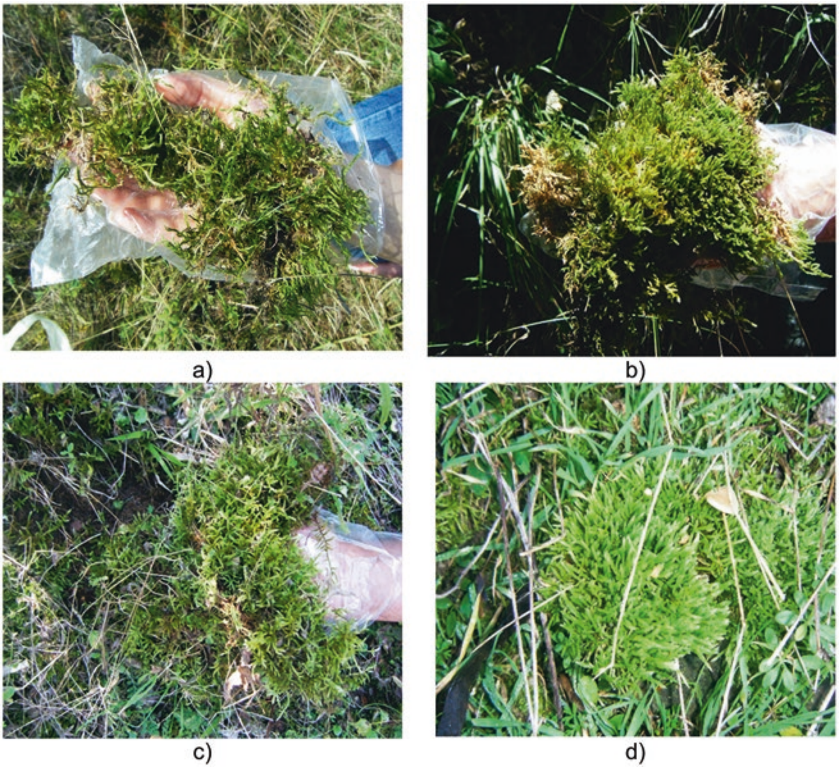


Fig. 3.2 Photos of the most present moss: (**a**) *Homalothecium lutescens* (Hedw. Robins), (**b**) *Hypnum cupressiforme* (Hedw.), (**c**) *Lencodon scinroides*, (**d**) *Scleropodium purum*

Large paper bags, tightly closed to prevent contamination, will be used during transportation. The amount of fresh moss needed for metal analysis is about 1 kg. The taken moss samples were dried on laboratory paper. The samples were then cleaned from dead material and attached litter, so that just the green and green-brown shoots from the 3 latest years were included.

Each sample was placed in a separate laboratory paper and turned several times until the moss was dried (room temperature 20–25 °C) to reach constant weight. The dried material that will not be used for planned analyses was stored in an environmental specimen bank for future investigations.

To avoid contamination, non-talcum plastic disposable gloves were used, and the mosses were handled on clean laboratory paper. No metal tools were used. For long-time storage, the moss samples were dried and put in sealed plastic bags together with information about the sample (species, locality name, coordinates, collecting date and other information that can be a subject of interest). The moss samples were stored in a dry place, at room temperature.

In total 107 moss samples were collected from the studied area. A map with the moss sampling locations is given in Fig. 3.3.

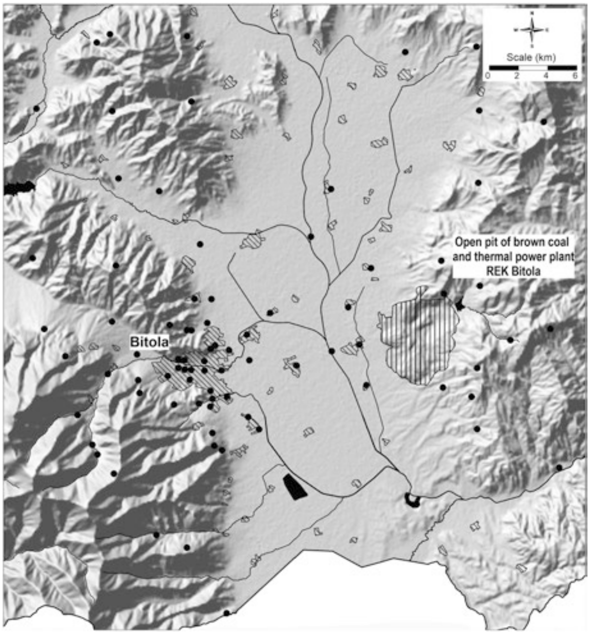


Fig. 3.3 The location in Bitola region for moss sampling

3.3.3 Digestion of Moss Samples

The moss sampling protocol was performed according to set standard rules for collection of such samples, as mentioned above. In the laboratory the samples were cleaned from extraneous plant material and air-dried at room temperature. Only the last 3 years' worth of growths of the moss materials was used without washing for the analysis.

Moss samples (0.5 g) were placed in Teflon digestion vessels, 5 mL concentrated nitric acid (HNO_3, 69%, trace pure for trace analysis) and 2 mL H_2O_2 (30%, *m/V* trace pure for trace analysis) were added, and the vessels were capped closed, tightened and placed in the rotor of the Mars microwave digestion system (Mars, CEM, USA). The moss samples were digested at 180 °C. After cooling, the digested samples were quantitatively transferred into 25 mL calibrated flasks as previously given by Balabanova et al. (2010) and Bačeva et al. (2012).

3.3.4 Analysis of Total Elements Contents

The investigated elements were analysed with the application of atomic emission spectrometry with inductively coupled plasma (AES-ICP). Atomic emission spectrometer with inductively coupled plasma (Varian 715-ES) was applied for the determination of 25 elements: Ag, Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Ga, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sr, V and Zn. For AES-ICP instrument calibration and quantitative determination of each element's contents in digests, a commercial standard mix solution (11355-ICP Multi Element Standard IV, Merck) was used. The correlation coefficient of calibration curve for each element was 0.999. In order to check for possible background contamination, blank samples were used and processed simultaneously with field samples. The method detection limit was calculated based on average measuring of the blank sample ($\gamma \pm 3\delta$). For all laboratory samples and standard solutions, treated ultra-pure water was used. The QC of the applied techniques was performed by standard addition method, and it was found that the recovery for the investigated elements ranges for ICP-AES between 98.5 and 101.2% and for ETAAS between 96.9% and 103.2%.

For this study, M2 and M3 moss samples certified reference materials (Steinnes et al. 1997) were used to check method accuracy, and for all considered elements, the difference between measured and certified values was within 15%.

Optimal conditions for the analysis of arsenic and cobalt by ETAAS and mercury by CV-AAS were given by Balabanova et al. (2010).

3.4 Results and Discussion

Data from the descriptive statistics of the measurements of 23 elements determined by ICP-AES (Ag, Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sr, V and Zn) in moss samples collected from 107 sampling locations in Bitola region are presented in Table 3.1. In Table 3.1 the following statistical parameters are given: *N*, number of samples; *X*, arithmetical average; *X*(BC), arithmetical average after Box-Cox method; Md, median; Min, minimum; Max, maximum; P_{10}, 10th percentile; P_{90}, 90th percentile; P_{25}, 25th percentile; P_{75}, 75th percentile; *S*, standard deviation; Sx, standard error; CV, coefficient of variation; *A*, skewness; *E*, kurtosis; BC, Box-Cox transformed values.

The values of macroelements Al, Ca, Fe, K, Mg and Na are in the following ranges: 0.008–1.6% Al; 0.02–1.9% Ca; 0.007–1.1% Fe; 0.009–1.2% K; 0.004–0.48% Mg and 0.003–0.26% P. High matrix content of these elements mostly originated from plant tissues, and it can be sometimes enriching the actual values from atmospheric distribution of these elements.

In Table 3.2 the comparison of the median, minimal and maximal values for the content of analysed elements in moss from Bitola region with those for the Republic of Macedonia and Europe is given. It could be seen that the median values for the content of almost all analysed elements in moss samples from Bitola region are very similar to those obtained for the Republic of Macedonia. The only difference is in the content of copper which in the moss samples from Bitola region is 3.24 times higher than that in the moss samples from the whole territory of Macedonia. If we compare the obtained results for the median value for the moss samples from the area around the thermoelectric power plant and those from wider region of Bitola, it could be seen that moss samples collected in the vicinity of REK Bitola have higher

Table 3.1 Descriptive statistics for the content of the elements in moss from Bitola region ($N = 107$)

Element	Unit	X	X(BC)	Md	Min	Max	P_{10}	P_{90}	P_{25}	P_{75}	S	S_X	CV	A	E	A(BC)	E(BC)
Ag	mg/kg	0.12	0.041	0.038	0.010	3.4	0.013	0.22	0.018	0.12	0.34	0.033	291	8.66	82.61	0.09	−0.92
Al	%	0.48	0.40	0.40	0.008	1.6	0.13	0.93	0.18	0.70	0.34	0.033	71	0.93	0.54	−0.05	−0.43
As	mg/kg	0.49	0.40	0.38	0.010	2.4	0.11	0.95	0.25	0.56	0.40	0.039	82	1.96	5.22	0.06	0.83
B	mg/kg	1.5	0.85	0.85	0.24	18	0.40	2.4	0.50	1.6	2.6	0.36	172	5.73	36.79	0.02	−0.03
Ba	mg/kg	51	50	48	1.1	130	23	84	36	67	24	2.3	47	0.39	0.27	−0.03	0.25
Ca	%	0.76	0.75	0.73	0.020	1.9	0.49	1.1	0.59	0.87	0.31	0.030	41	0.99	3.36	0.29	2.97
Cd	mg/kg	0.10	0.046	0.051	0.010	1.9	0.012	0.12	0.032	0.077	0.25	0.024	241	5.78	36.22	−0.05	0.65
Co	mg/kg	1.2	0.86	0.83	0.010	6.6	0.19	2.7	0.40	1.6	1.1	0.11	95	1.99	5.41	−0.01	0.23
Cr	mg/kg	6.2	5.0	4.9	0.13	22	1.6	14	2.6	8.2	4.8	0.47	77	1.10	0.61	−0.03	−0.35
Cu	mg/kg	9.9	8.0	8.1	0.24	44	2.7	19	4.0	13	8.0	0.77	80	1.86	4.70	0.02	0.30
Fe	%	0.41	0.35	0.36	0.007	1.1	0.12	0.82	0.18	0.59	0.28	0.027	68	0.78	−0.21	−0.06	−0.46
K	%	0.48	0.47	0.45	0.009	1.2	0.28	0.79	0.34	0.58	0.22	0.021	45	0.83	1.58	0.14	1.50
Li	mg/kg	2.3	1.8	1.9	0.040	9.7	0.58	5.0	0.83	3.4	1.9	0.18	83	1.48	2.32	−0.01	−0.08
Mg	%	0.26	0.25	0.24	0.004	0.48	0.11	0.41	0.16	0.38	0.12	0.012	47	−0.03	−1.03	−0.22	−0.76
Mn	mg/kg	150	140	130	3.5	620	48	260	90	210	96	9.3	63	1.58	5.30	0.06	1.07
Mo	mg/kg	0.64	0.38	0.32	0.010	3.3	0.010	1.6	0.15	0.91	0.71	0.069	111	1.75	3.00	−0.06	−0.43
Na	mg/kg	130	110	100	2.7	540	65	340	76	140	100	10	77	2.43	6.59	0.14	2.55
Ni	mg/kg	4.6	4.1	4.0	0.050	17	1.8	8.6	2.4	5.9	3.0	0.29	65	1.31	2.35	0.07	0.69
P	%	0.12	0.12	0.12	0.003	0.26	0.064	0.20	0.086	0.16	0.053	0.005	43	0.18	−0.11	−0.06	0.07
Pb	mg/kg	3.9	2.8	2.8	0.040	28	0.83	9.0	1.7	4.1	4.3	0.41	108	2.99	11.87	0.08	1.16
Sr	mg/kg	27	26	26	0.64	81	13	45	20	33	13	1.2	47	0.82	2.16	0.10	1.32
V	mg/kg	7.1	5.8	5.7	0.16	23	1.7	16	2.6	9.9	5.4	0.52	76	1.01	0.31	−0.04	−0.43
Zn	mg/kg	33	24	23	0.73	400	8.3	59	15	37	43	4.2	132	6.30	50.58	0.11	3.05

N number of samples analysed, *X* arithmetical average, *X(BC)* arithmetical average after Box-Cox method, *Md* median, *min* minimum, *max* maximum, P_{10} 10th percentile, P_{90} 90th percentile, P_{25} 25th percentile, P_{75} 75th percentile, *S* standard deviation, S_x standard error, *CV* coefficient of variation, *A* skewness, *E* kurtosis, *BC* Box-Cox transformed values

Table 3.2 Comparison of the median, minimal and maximal values for the content of analysed elements in moss from Bitola region with those for the Republic of Macedonia and Europe

Element	Unit	Bitola region (this study)		Area around TEP Bitola (this study)		North Macedonia, 2015 (Stafilov et al. 2018b)		Europe (Harmens et al. 2008)	
		Md	Min–Max	Md	Min–Max	Md	Min–Max	Md	Min–Max
Ag	mg/kg	0.04	0.01–3.4	0.06	0.01–0.37	–	–	–	–
Al	%	0.40	0.080–1.6	0.54	0.062–1.5	0.21	0.075–0.74	–	–
As	mg/kg	0.38	0.01–2.4	0.50	0.09–1.53	0.54	0.13–1.4	0.42	0.10–9.36
B	mg/kg	0.85	0.24–18	0.67	0.48–1.62	–	–	–	–
Ba	mg/kg	48	1.1–130	48	7.2–87	42	9.7–180	–	–
Ca	%	0.73	0.020–1.9	0.73	0.072–1.9	0.69	0.35–1.30	–	–
Cd	mg/kg	0.05	0.01–1.9	0.05	0.01–0.14	0.23	0.018–0.88	0.20	0.07–1.26
Co	mg/kg	0.83	0.01–6.6	0.75	0.19–2.82	0.60	0.16–2.0	–	–
Cr	mg/kg	4.9	0.13–22	6.3	1.74–19	5.7	1.8–31	2.32	0.72–29.3
Cu	mg/kg	8.1	0.24–44	12.5	2.15–39	4.6	3.0–8.3	6.80	3.07–91.2
Fe	%	0.36	0.007–1.1	0.44	0.05–1.1	0.17	0.051–0.46	0.08	0.023–0.61
K	%	0.45	0.009–1.2	0.40	0.04–0.62	0.60	0.31–1.4	–	–
Li	mg/kg	1.9	0.040–9.7	2.6	0.21–9.7	0.79	0.32–3.5	–	–
Mg	%	0.24	0.004–0.48	0.24	0.019–0.39	0.19	0.12–0.38	–	–
Mn	mg/kg	130	3.5–620	117	22–257	160	33–510	–	–
Mo	mg/kg	0.32	0.010–3.3	0.74	0.11–2.4	0.17	0.085–0.51	–	–
Na	mg/kg	100	2.7–540	107	61–526	190	140–380	–	–
Ni	mg/kg	4.0	0.050–17	4.9	1.10–12	3.5	0.68–63	2.26	0.71–63
P	%	0.12	0.003–0.26	0.13	0.019–0.21	–	–	–	–
Pb	mg/kg	2.8	0.040–28	2.9	0.65–11	4.9	2.2–14	1.76	1.76–46.9
Sr	mg/kg	26	0.64–81	28	0.65–50	25	6.5–220	–	–
V	mg/kg	5.7	0.16–23	7.1	0.59–22	3.3	0.47–11	2.82	0.80–22
Zn	mg/kg	23	0.73–400	27	4.7–400	30	12–66	34	15–177

Md median, *Min* minimum, *Max* maximum

content of several elements: arsenic, chromium, copper, molybdenum, nickel and zinc. This can be explained by higher content of these elements in fly ash than in the soil from this area. This indicates that the distribution of fly ash from the dump site and from the emission of dust from the chimneys influences the air pollution with small particles and by the spatial distribution of these elements, it could be determined the size of the area affected by dust from REK Bitola.

The ratios of the average content of the analysed elements in top soil (T) according to data from Stafilov et al. (2018a) and moss samples (M) are presented in Table 3.3. It could be seen that despite the elements (Ba, Ca, Mg, Mn) present in the surrounding soils, higher correlation was found also for the copper and zinc which are present in higher content in the polluted soil around the REK Bitola.

The matrix of correlation coefficients between all of the analysed elements is given in Table 3.4. By applying bivariate statistics, the degree of correlation between the analysed elements in the soil samples was determined. It was usually assumed that the absolute value of the correlation coefficient between 0.3 and 0.7 indicates a

Table 3.3 The ratio of the element average content in top soil (T) samples and moss samples (M)

Element	T/M
Al	−0.03
As	0.09
B	**0.54**
Ba	0.23
Ca	0.20
Cd	0.05
Co	0.01
Cr	−0.04
Cu	**0.41**
Fe	−0.07
K	0.22
Li	0.16
Mg	0.36
Mn	0.18
Na	−0.05
Ni	0.05
P	0.08
Pb	0.02
Sr	0.10
V	0.01
Zn	**0.25**

Bolded values show high correlation

good association and between 0.7 and 1.0 points to the existence of a strong connection between elements. The values for the content of every element were correlated with the values for the content of the other elements (Reimann et al. 2002). All correlation coefficients between all elements are represented in the matrix of correlation coefficients (Table 3.4).

In factor analysis the distribution is reduced to four synthetic variables (F1 to F4). As it can be seen from the results obtained by factor analysis (Table 3.5), four factors (i.e. associations of elements) were obtained: F1 (Fe, Al, V, Ni, Li, Cr, Co, Ba, Pb, S and Mn), F2 (Cu, Mo, Ag and Zn), F3 (K, P, Na, Mg and Ca) and F4 (As and Cd). A total of 23 elements were reduced by factor analysis to 22 elements, which had a total factor loading of 74.9% of the variability (5). Boron was eliminated from further analysis because it had a low share of communality or low tendency to form independent factors.

The dendrogram obtained by the application of cluster analysis is given in Fig. 3.4. In the dendrogram the elements are divided into two major clusters and several sub-clusters according to the degree of correlation between them. The first sub-cluster includes the elements Ag, Cu and Mo which are included in Factor 2 of the matrix of loading of the dominant rotating factors (Table 3.5). The second sub-cluster is composed of Al, Fe, Cr, V, Li, Ni, Sr, Zn, Na, Co, Mn and Pb which belong to Factor 1 of the factor analysis. The third sub-cluster consists of As and Cd which belong to Factor 4, and the fourth sub-clusters include Ca, K, P, Mg and Na which belong to Factor 3.

Table 3.4 Matrix of correlation coefficients ($n = 107$)

Ag	1.00																				
Al	0.23	1.00																			
As	0.07	0.20	1.00																		
B	0.03	−0.03	0.27	1.00																	
Ba	0.03	**0.72**	0.26	−0.04	1.00																
Ca	0.12	0.63	0.31	0.11	0.62	1.00															
Cd	−0.12	0.05	0.50	0.42	0.14	0.13	1.00														
Co	0.14	**0.76**	0.37	0.05	0.65	0.56	0.22	1.00													
Cr	0.40	**0.91**	0.15	0.05	0.61	0.63	−0.04	**0.70**	1.00												
Cu	0.61	0.38	0.09	0.37	0.17	0.30	−0.09	0.14	0.58	1.00											
Fe	0.21	**0.98**	0.19	0.02	**0.71**	0.65	0.05	**0.79**	**0.93**	0.40	1.00										
K	0.02	0.36	0.17	0.13	0.56	0.56	0.13	0.35	0.31	0.10	0.38	1.00									
Li	0.32	**0.92**	0.26	−0.02	0.64	0.61	0.07	**0.71**	**0.85**	0.47	**0.90**	0.28	1.00								
Mg	−0.35	0.37	0.22	−0.22	0.62	0.54	0.21	0.47	0.24	−0.26	0.40	0.67	0.27	1.00							
Mn	0.21	0.59	0.23	−0.29	0.64	0.47	−0.01	0.59	0.54	0.25	0.60	0.33	0.57	0.41	1.00						
Mo	0.54	0.34	0.10	0.32	0.13	0.31	−0.16	0.19	0.52	**0.70**	0.35	0.14	0.41	−0.15	0.17	1.00					
Na	0.17	0.50	0.22	0.05	0.55	0.56	0.01	0.43	0.50	0.34	0.50	0.64	0.49	0.41	0.42	0.34	1.00				
Ni	0.22	**0.84**	0.28	−0.03	0.63	0.65	0.11	**0.78**	**0.86**	0.34	**0.87**	0.34	**0.80**	0.43	0.68	0.31	0.48	1.00			
P	0.17	0.31	0.17	0.19	0.49	0.64	0.09	0.30	0.36	0.28	0.35	**0.74**	0.28	0.48	0.34	0.33	0.57	0.35	1.00		
Pb	0.12	0.56	0.15	−0.09	0.48	0.48	0.22	0.49	0.56	0.27	0.58	0.12	0.55	0.21	0.42	0.13	0.25	0.62	0.19	1.00	
Sr	0.32	0.69	0.12	0.12	**0.70**	0.61	−0.05	0.53	**0.73**	0.49	0.69	0.39	0.66	0.34	0.53	0.48	0.49	0.61	0.36	0.41	1.00	
V	0.32	**0.94**	0.17	0.07	0.64	0.64	0.00	**0.75**	**0.97**	0.52	**0.97**	0.32	**0.90**	0.28	0.58	0.46	0.50	**0.88**	0.34	0.57	**0.73**	1.00
Zn	0.53	0.64	0.11	−0.07	0.49	0.57	−0.02	0.52	**0.77**	0.67	0.66	0.36	0.67	0.12	0.51	0.54	0.54	0.66	0.44	0.53	0.69	**0.75**
	Ag	**Al**	**As**	**B**	**Ba**	**Ca**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**K**	**Li**	**Mg**	**Mn**	**Mo**	**Na**	**Ni**	**P**	**Pb**	**Sr**	**V**

Bolded values show high correlation

Table 3.5 Matrix of dominant rotated factor loadings ($n = 107$, 22 selected elements)

Element	F1	F2	F3	F4	Comm
Fe	**0.94**	0.11	0.21	−0.05	93.7
Al	**0.93**	0.12	0.18	−0.04	90.9
V	**0.92**	0.26	0.15	−0.09	94.0
Ni	**0.88**	0.06	0.19	0.10	82.3
Li	**0.87**	0.21	0.09	0.03	80.8
Cr	**0.86**	0.37	0.16	−0.11	91.4
Co	**0.78**	−0.12	0.20	0.24	72.5
Ba	**0.68**	−0.13	0.52	0.04	74.5
Pb	**0.65**	−0.01	−0.05	0.23	48.3
Sr	**0.64**	0.31	0.31	−0.20	64.8
Mn	**0.58**	−0.02	0.24	0.00	39.2
Cu	0.22	**0.86**	0.02	−0.08	80.0
Mo	0.17	**0.82**	0.15	−0.09	73.1
Ag	0.02	**0.80**	−0.10	−0.02	64.8
Zn	0.54	**0.57**	0.28	−0.09	70.2
K	0.11	−0.10	**0.90**	0.00	83.9
P	0.07	0.15	**0.86**	0.07	76.9
Na	0.29	0.17	**0.69**	−0.03	59.1
Mg	0.29	−0.57	**0.66**	0.03	84.2
Ca	0.50	0.05	**0.65**	0.08	67.5
As	0.10	0.04	0.10	**0.87**	77.4
Cd	−0.01	−0.23	−0.02	**0.85**	77.3
Prp.Totl	36.1	16.5	14.5	7.7	74.9
EigenVal	9.64	3.30	2.08	1.44	
Expl.Var	7.93	3.20	3.64	1.70	

F1, F2, F4 factor loadings, *Var* variance (%), *Com* communality (%), *Prp.Tol* total amount of the explained system variance, *Expl.Var* particular component variance, *EigenVal* eigenvalue
Bold values indicate affiliation to the corresponding factor

To construct the maps for the distribution of the elements as well as for the factor associations, the universal kriging method was applied. Kriging is an optimal prediction method, indented for geophysical variables with continuous data distribution. The obtained values of variables can sometimes be random, but their variance is not described with geometric function. This method performs projection of an object by using the values of certain parameters that describe its position (latitude, longitude and oval height), i.e. any object can be spatially defined. All data about variables are organised in several possible ways; most common one used is raster. Generally, raster consists of a matrix of cells (pixels) arranged in rows and columns, where each cell contains values that provide information about variables. Digital air photography, satellite imagery, digital images and scanned maps can be used as a raster. Kriging interpolation performs the output of each raster cell by calculating the average load of nearby vectors. Kriging method analyses the statistical variation of the values of different distances and at various positions and determines the shape and size of the specified point for examination as a set of load factors.

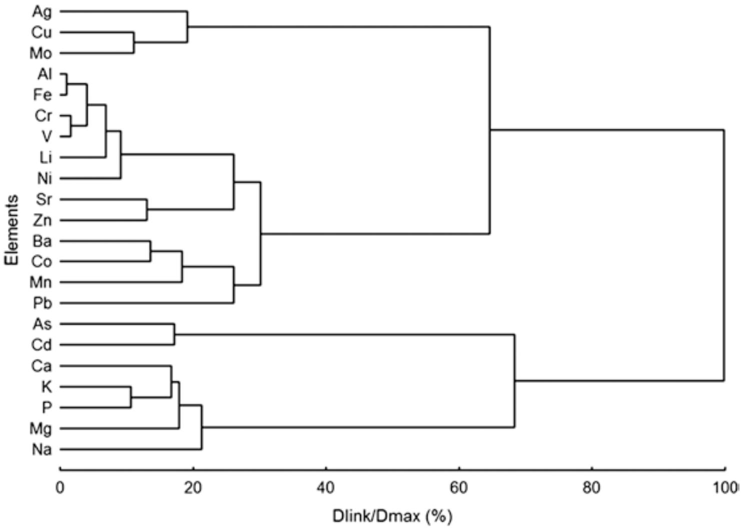


Fig. 3.4 Dendrogram from the cluster analysis of moss analysis

For the development of the distribution maps, the kriging method with linear variogram interpolation was applied. As area limits, we considered the percentile values of the distribution of the interpolated values. The following seven areas of percentile values were selected: 0–10, 10–25, 25–40, 40–60, 60–75, 75–90 and 90–100. The distribution maps for the individual elements are prepared in seven concentration ranges for each element with different colour.

Factor 1 (Fe, Al, V, Ni, Li, Cr, Co, Ba, Pb, Sr and Mn) The spatial distribution map of the factor scores of factor F1 (Fig. 3.5) shows that these elements are present in varying concentrations throughout the studied area. Higher contents of these elements are found in the soil samples from the central part of the study where Quaternary sediments are dominant geological formations as well as on the mountain Baba where Paleozoic schist and granites prevailed (Fig. 3.5). From the list of the elements, it could be expected that some of the elements associated in Factor 1 have higher content in the area close to TEP Bitola and in the area between TEP Bitola and the city of Bitola. This is visible from the maps of spatial distribution of all elements included in Factor 1 which are presented in Fig. 3.6a and 3.6b. This particularly applies to the spatial distribution of chromium (Fig. 3.6a) and zinc (included in Factor 2) (Figs. 3.7 and 3.8) which are present in higher content in fly ash than in the surrounding soil.

Factor 2 (Cu, Mo, Ag and Zn) The spatial distribution map of the factor scores of factor F2 (Fig. 3.7) shows that these elements are present in higher contents in the southern part of the study area where Quaternary and Pliocene sediments are domi-

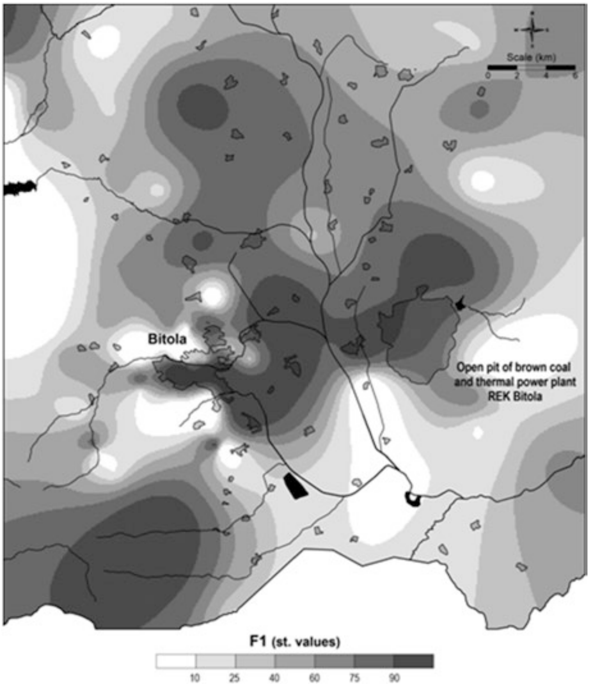


Fig. 3.5 Spatial distribution of Fe, Al, V, Ni, Li, Cr, Co, Ba, Pb, Sr and Mn

nant geological formations (Stafilov and Šajn 2016, 2019). From the list of the elements, it could be expected that some of the elements associated in Factor 2 have higher content in the area close to REK Bitola and in the area between REK Bitola and the city of Bitola. It is visible from the maps of spatial distribution of all elements included in Factor 1 which are presented in Fig. 3.8. This particularly applies to the spatial distribution of Zn, Cu and Mo (Fig. 3.8) which are present in higher content in fly ash than in the surrounding soil.

Factor 3 (K, P, Na, Mg and Ca) The spatial distribution map of the factor scores of factor F3 (Fig. 3.9) shows that these elements are present in higher contents in the northern-central part of the study area where Quaternary and Pliocene sediments are dominant geological formations (Fig. 3.9). From the list of the elements, it could be expected that some of the elements associated in Factor 3 have higher content in the area close to REK Bitola and in the area between REK Bitola and the city of Bitola. It is visible from the maps of spatial distribution of all elements included in Factor 1 which are presented in Fig. 3.10. This particularly applies to the spatial distribution of copper (Fig. 3.10) which is present in higher content in fly ash than in the surrounding soil.

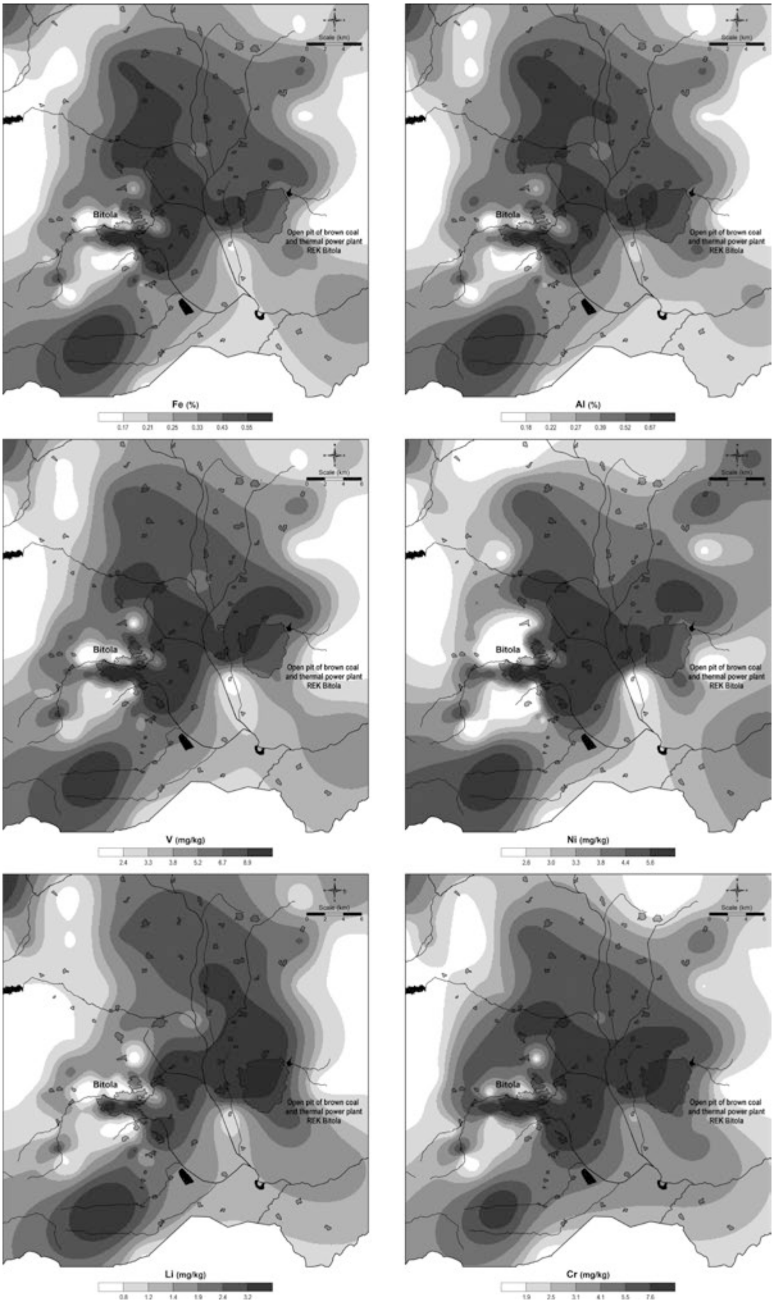


Fig. 3.6 (**a**) Spatial distribution of Fe, Al, V, Ni, Li and Cr. (**b**) Spatial distribution of Co, Ba, Pb, S and Mn

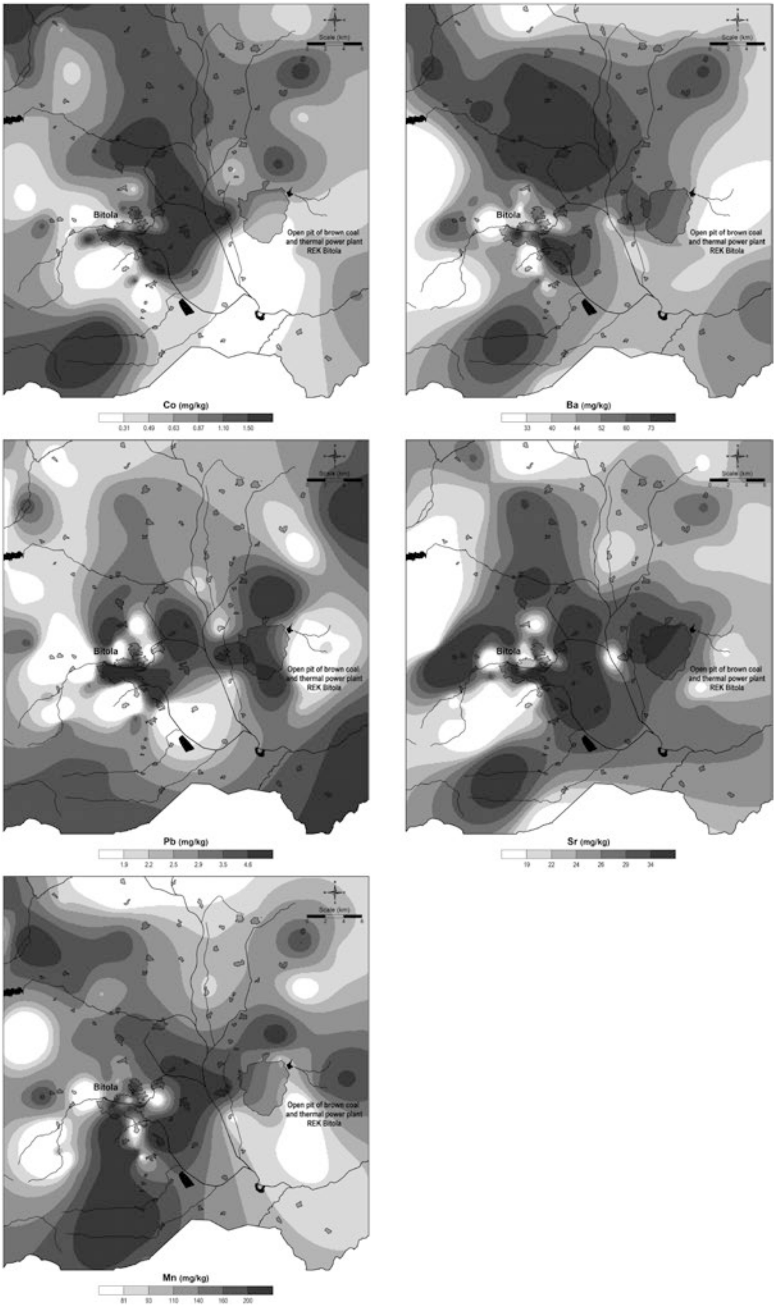
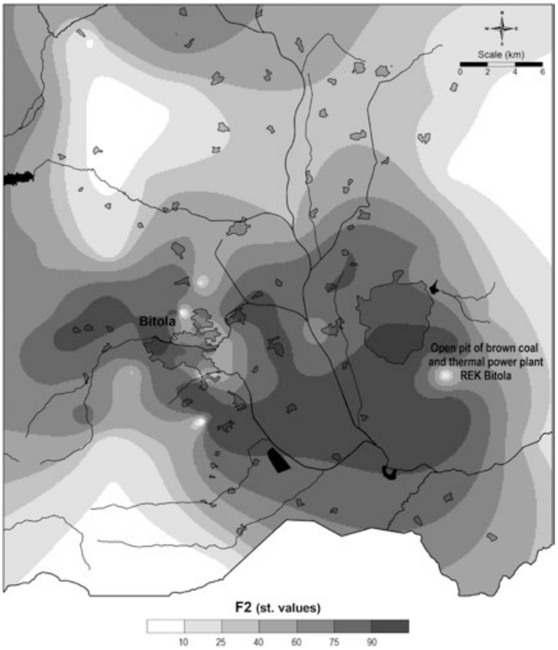


Fig. .3.6 (continued)

Fig. 3.7 Spatial distribution of Factor 2 scores (Cu, Mo, Ag and Zn)



Factor 4 (As, Cd) The spatial distribution map of the factor scores of factor F4 (Fig. 3.11) shows that these elements are present in higher contents in the mountainous areas of the study area, such as mountain Baba (with dominant Paleozoic schists and granites) on the west and Selečka Mountain on the west (with dominant Proterozoic gneisses and granites). From the list of the elements, it could be expected that some of the elements associated in Factor 3 have higher content in the area close to REK Bitola and in the area between REK Bitola and the city of Bitola. It is visible from the maps of spatial distribution of all elements included in Factor 1 which are presented in Fig. 3.12. This particularly applies to the spatial distribution of copper (Fig. 3.12) which is present in higher content in fly ash than in the surrounding soil.

3.5 Conclusion

From the results of the air pollution study in the Bitola region by moss biomonitoring, it could be concluded that the analysed elements are associated into four associations of elements: Factor 1 (Fe, Al, V, Ni, Li, Cr, Co, Ba, Pb, Sr and Mn), Factor 2 (Cu, Mo, Ag and Zn), Factor 3 (K, P, Na, Mg and Cu) and Factor 4 (As, Cd). The elements included in the first association are present in varying concentrations

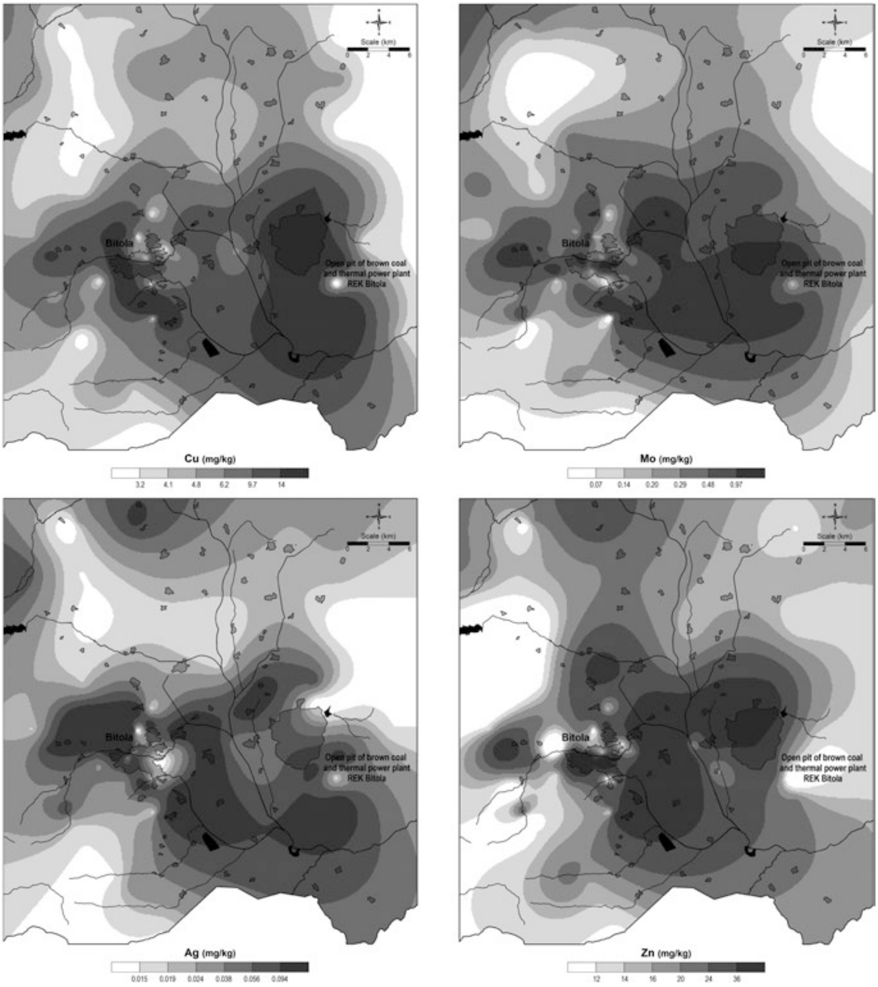


Fig. 3.8 Spatial distribution of Cu, Mo, Ag and Zn

throughout the studied area with higher contents of these elements found in the moss samples from the central part of the study area. It was found that some of the elements have higher content in the area close to REK Bitola and in the area between REK Bitola and the city of Bitola. This is the case for the spatial distribution of chromium and zinc as well as for zinc and copper (from Factors 2 and 3). The spatial distribution maps indicate the areas under the dust from the fly ash and slag deposit in the REK Bitola because these elements are present in higher content in this material than in the surrounding soil.

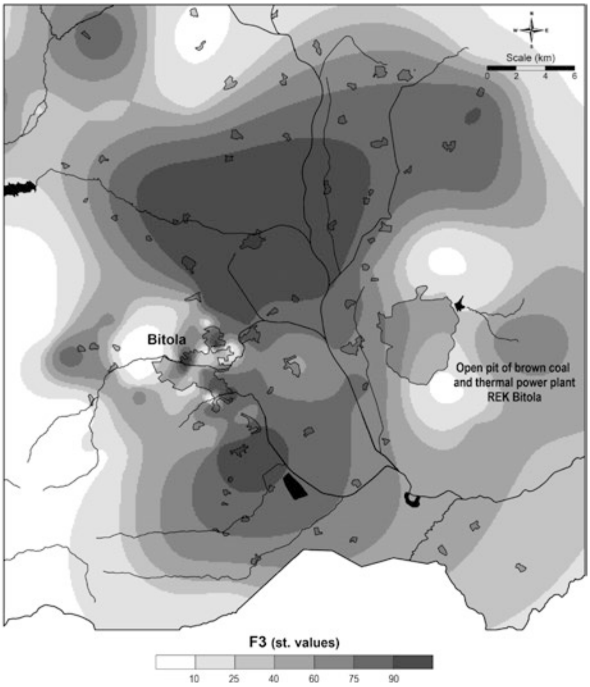


Fig. 3.9 Spatial distribution of Factor 3 scores (K, P, Na, Mg and Ca)

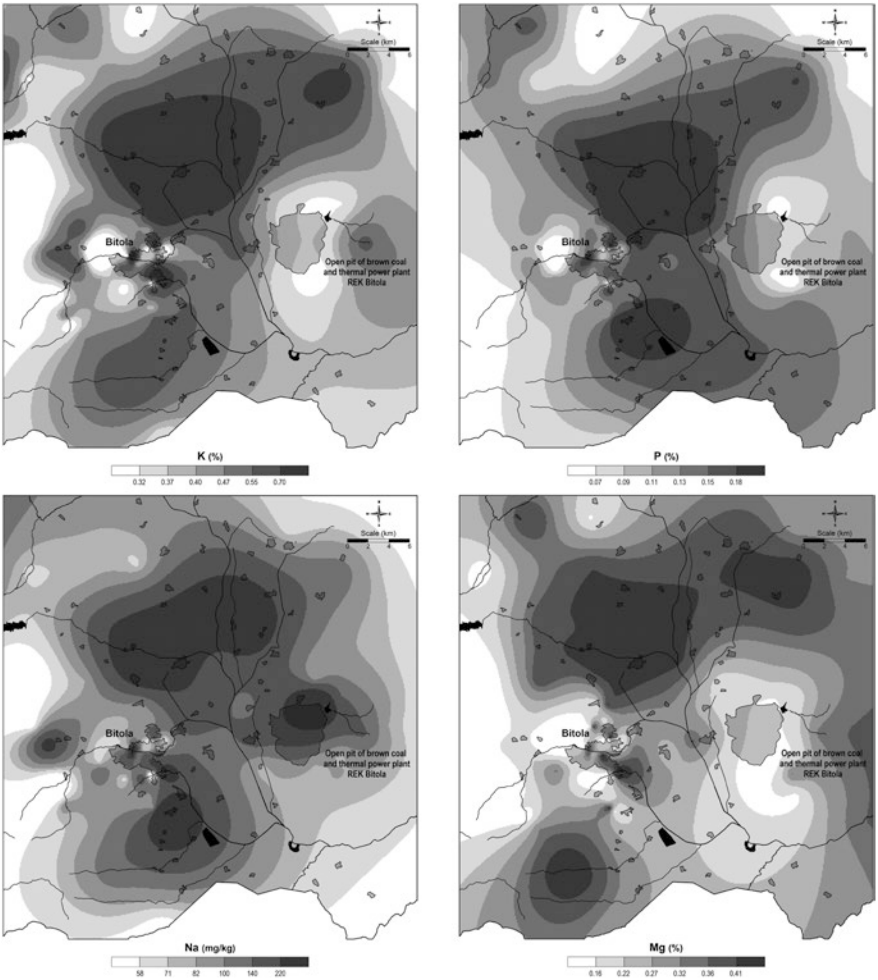
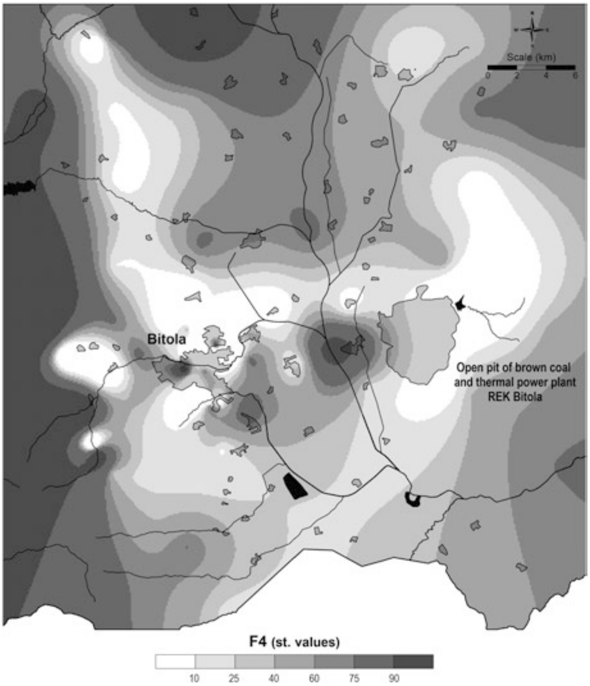


Fig. 3.10 Spatial distribution of K, P, Na, Mg and Ca

Fig. 3.11 Spatial distribution of Factor 4 scores (As and Cd)



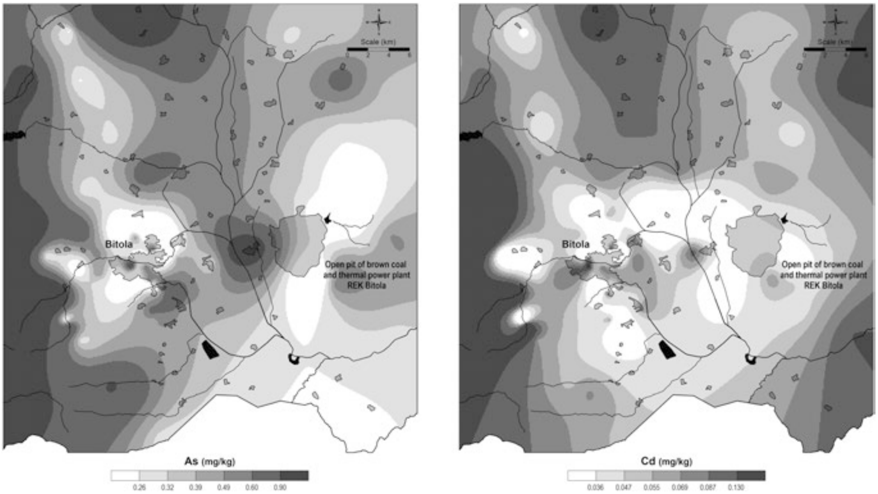


Fig. 3.12 Spatial distribution of arsenic and cadmium

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Chapter 4
Lichens as the Main Indicator in Biological Monitoring of Air Quality

Svetlana Ristić, Robert Šajn, and Slaviša Stamenković

Abstract Based on the available knowledge on air quality impairment, there is now a need to know the type and degree of pollution. One of the basic prerequisites for improving air quality is the timely detection of pollution. The bioindication method is widely used as a significant tool for air quality assessment by taking into account the temporal dimension of the negative impact of pollutants in the ecosystem based on the reaction of living organisms to the total sum of all the effects of different environmental factors. This is the only method that assesses the impact of air quality on living beings directly. The best bioindicators are lichens due to their pronounced sensitivity to air quality changes. Over time, lichens accumulate a variety of different pollutants which may pose potential risks to ecosystems and human health. Taking into account the time dimension, it is especially useful to monitor the state of air quality in anthropogenically modified ecosystems. Therefore, the focus of this chapter is the indication and zoning of different degrees of air quality in selected seminatural and anthropogenically modified ecosystems of Serbia over a longer period of time, based on the contemporary methods of using lichens as bioindicators.

Keywords Passive biomonitoring · Lichens · Air pollution · Spatial distribution · Indication zone

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4.1 Introduction

Air is a mixture of gases necessary for the life of all living beings. The significance of air quality is very important for the proper and undisturbed development of terrestrial and aquatic organisms. However, in the case of a qualitative or quantitative change in the physical, chemical, or biological characteristics of the air, which leads to the violation and disruption of the known laws of ecosystem functioning, then it is air pollution. Furthermore, if specific substances are released into the air, most often from various processes of anthropogenic origin and which are not its integral part, pollution occurs. Air pollution can also occur if the usual concentrations of pre-existing substances in the air rise to a level higher than the upper point of the amplitude of variation of a given substance. As a rule, air pollution is not a phenomenon of a local character as it can easily be transmitted over smaller or larger distances in relation to the place of the source of the pollution.

Air pollution can be natural and anthropogenic. The group of natural sources of air pollution includes pollutants of biological origin, plant, animal, and microbial, as well as inorganic pollutants: volcanic gasses, forest fires, thermal source gases, electrical discharges in the atmosphere, etc. Anthropogenic sources of air pollution are the result of various forms of human activities in the areas of industry, technology, transportation, and production of electricity (Caggiano et al. 2017).

The degree of air pollution is more or less directly or indirectly affected by climatic and meteorological conditions, urban solutions, terrain configuration, etc. as well as the distribution of pollutants in the air, which changes spatially and temporally (Llop et al. 2012; Tilmes et al. 2017). The difference between natural and anthropogenic air pollution is reflected in the amount and type of pollutants. In addition, natural air pollution is occasionally localized, while anthropogenic air pollution is of permanent and global character (Marcilio and Gouveia 2007). Anthropogenic air pollution has arisen and escalated in a short period of time and has produced more or less significant consequences for the living world in general. Anthropogenic pollution is characterized by the presence of some pollutants such as carbon monoxide (CO), carbon dioxide (CO_2), sulfur dioxide (SO_2), and nitrogen oxides (NOx).

Carbon monoxide is one of the gasses whose emissions mainly originate from traffic. Carbon dioxide is a gas whose amount has increased dramatically in recent years, with a tendency for further growth. This increase in CO_2 comes from the burning of fossil fuels (Pearson 2000). The increase in the amount of CO_2 in the atmosphere plays a significant role in global warming and the formation of the greenhouse effect. Global warming would have crucial consequences for all life on the planet.

Sulfur dioxide is emitted into the atmosphere every year; however, with the adoption of international legislations in recent years, the concentration of this gas is significantly being reduced (Aas et al. 2019). Due to the increased combustion of fossil fuels, the emission of this gas is significantly increased during the winter months (Rubio-Salcedo et al. 2017). The presence of SO_2 in the atmosphere serves as a cause for the appearance of acid rains.

Nitrogen oxides also cause the appearance of acid rains. Furthermore, these gases have an irritating effect on the respiratory organs, and they manifest their harmfulness by causing photochemical smog. Important components of the photochemical smog are also hydrocarbons, which end up in the atmosphere mostly as components of car exhaust gases (Boltersdorf and Werner 2013).

The negative effects of air pollution are seen at all levels of the biological organization of living beings (Guttová et al. 2011). Many natural values are endangered. Many plants are subjected to types of permanent damage named "early aging." The consequences of air pollution are also reflected in the climate (Evju and Bruteig 2013). Suspended particles of mater in the polluted air decrease visibility and reduce sunlight by 15–20%, resulting in a decrease in temperature.

An increase in the amount of pollutants in the air can sometimes have greater or lesser consequences on human health, as it affects the deterioration of the condition of people suffering from various diseases, especially respiratory and heart diseases, such as lung and bronchus cancer, anemia, respiratory irritations, acute lung function problems, a decrease in the vital capacity of children living in cities and industrial zones, an increase in the number of people suffering from diabetes, etc. Even short-term exposure to sulfur and nitrogen oxides can lead to breathing difficulties due to the formation of edema, mucus, and bronchial spasm, as a result of irritation and inflammation (Wu et al. 2016).

Based on the available knowledge of air quality hazards, a need to know the type and the degree of threat has arisen (Hauck et al. 2013). One of the fundamental preconditions for improving the air quality is a timely detection and registration of the increased levels of air pollution, i.e., organizing a monitoring system. A monitoring system is a system of successive observations of the elements of the environment in space and time. Monitoring system is reflected in the collection of data of a quantitative and qualitative nature on the presence and distribution of pollutants in space and time, the monitoring of emissions and immissions of pollution sources and their effect, the transportation of pollutants, and the special role of this system is to monitor the effect pollution has on living beings (Wolterbeek 2002).

Two groups of monitoring methods have been developed:

1. Physicochemical methods of monitoring
2. Biological methods of monitoring

Physicochemical monitoring methods are based on physicochemical measurement and calculation of the amount and concentration of individual pollutants in the air, in a unit volume, in a unit of time, or in mathematical modeling (cybernetics) and predicting changes in environmental quality (Samanta et al. 2002).

Biological monitoring methods are based on registering and monitoring reactions and changes that occur under the influence of pollutants at some level of the biological organization of living beings (Paoli et al. 2015). It is precisely these different levels that are seen as specific targets for the effects of the pollutants.

Biological monitoring methods show certain advantages compared to their physicochemical counterparts. Physicochemical methods reflect the cross section of the current state of affairs, and it is not possible to gain an objective insight (and predict the true effect) of the complex impact various pollutants have on living beings at the place where they live.

In contrast, bioindication methods perceive the time dimension of the negative effects of pollutants in the ecosystem and are based on the response of living beings to the simultaneous sum (synergism) of all the influences of different environmental factors (Das et al. 2013). Bioindication methods lead to results and conclusions which speak of the cumulative effect of long-term influence of different (small, but rising) concentrations of pollutants in the air, which cannot be detected by methods of physical and chemical measurement of air pollution (Wolterbeek 2002). The bioindication method most often uses the indicator organism as a pollution-detecting instrument. In this regard, there are two types of monitoring the response of the organism indicator to the changed environmental conditions: passive biomonitoring and active biomonitoring. Passive biomonitoring means monitoring organisms in places where they are naturally present (natural habitats), while active biomonitoring involves the use of indicator organisms as transplants. Moss, lichen, or some vascular plants can be used as indicator organisms (Balabanova et al. 2017; Aničić Urosević et al. 2020). The best bioindicators of air quality are lichens (Loppi 2014; Will-Wolf et al. 2015).

Lichens are symbiotic organisms made up of fungi, most commonly from the *Ascomycotina subphylum*, and algae from the divisions Cyanobacteriophyta or Chlorophyta (Nash 2008). In this structure, algae and fungi have changed so much and lost their independence that they can no longer be found as such freely in nature. Both members benefit from this symbiosis as they complement each other and enable the others' survival. The algae perform photosynthesis and supply the fungus with organic matter, while the fungus absorbs the mineral salt-rich water from the substrate with the help of hyphae. The exchange of matter between the algae and the fungus is done through their cell walls. The fungi are regarded as lichen builders as they predominate in quotative terms (Gadd 2011).

Lichens are very sensitive to the changes in air quality. They do not possess the protective surface layer on their thallus, so the pollutants can breach the inside of the lichen thallus uninterrupted. Lichens feed by absorbing the nutrients from the air (water vapor) and, to a lesser extent, from the substrate. The level of lichen metabolism increases when they are in a hydrated state, so in such a state, they are more sensitive to the quality of the air than when they are dry. The hydrated state occurs just in time when precipitations "wash away" harmful substances from the air and bring them to the thallus of the lichens. Lichens do not have the ability of shedding individual body parts in the manner of which vascular plants shed their leaves, thus getting rid of accumulated harmful substances. The accumulation of harmful substances in the thallus of the lichens is favored by the fact that lichens do not have the ability of excretion, so whatever they adopt, it remains permanently in their bodies. Lichens are characterized by very slow growth due to them metabolizing throughout the year. The negative influences of harmful substances cannot be quickly remedied, so it is possible to monitor them for an extended period of time.

Some terricolous (on land) and saxicolous (on stone, rock) lichen species (*Micarea confusa, Collemopsidium chlorococcum, Coppinsia minutissima*) have also proven themselves to be very good indicators of soil pollution caused by heavy metals (Coppinis and Van den Boom 1995; Aptroot and Van den Boom 1998; Lumbsch and Heibel 1998).

Comparative measurements of nitrogen concentration (N deposition) in the tissues of three bioindicator organisms – moss, lichen, and the bark of 18 different plant species – have shown that all of these organisms, and especially lichens, are very good indicators of nitrogen pollution and that they are cheap alternatives to the expensive N waste measurement stations installed in Germany (for deposition measurements in Germany) (Boltersdorf et al. 2014).

4.2 Method of Lichen Indication of Air Quality

The sensitivity of lichens to the changes in air quality was noticed as early as 1866. Nylander was the first to notice the property of lichens to react to harmful substances in the air, and he attributed this to the influence of factory smoke (Nylander 1866). Namely, it was noticed that the complete disappearance of lichens from the Luxembourg Park in Paris is positively correlated with the expansion of the replacement of wood with coal as an energy source for heating households. It was noticed that, in the 1970s and 1980s, in areas known for reduced air quality, lichens have either completely disappeared or have had their composition radically altered. After that, various analyses have pointed to the connection between air pollution and lichen composition (Mayer et al. 2013), so scales of lichen resistance to air pollution were formed. First such scale was formed by Hawksworth and Rose (Hawksworth and Rose 1970).

During the nineteenth century, there was a decrease or complete disappearance of lichens from urban parts of the cities. Immediately after that, independent researches were carried out in England, Munich, and Paris with the goal of investigating the so-called European phenomenon of lichen disappearance (Nylander 1866; Arnold 1900). It was noticed that, as a rule, in the city center, the number of individual lichen as well as types of lichens is negligibly small in relation to the environment, while in some industrial zones, lichens are completely absent. Such observations have been linked to air pollution due to an increase in sulfur dioxide concentrations. It has been experimentally proven that SO_2 (as one of the best studied pollutants) already in a concentration of 0.08–0.1 mg/m^3 of air begins to have a negative effect on lichens, because it leads to the formation of brown fields in algae chloroplasts which, after some time, causes necrosis and decay of the entire lichen (Nash and Gries 2002). It has been established that, in the conditions of higher levels of SO_2 content as well as soot in the air, the rate of morbidity of the population from respiratory diseases has increased, which can lead to death. Heart attack cases were registered in parallel, as a result of a link between elevated SO_2 content and a higher number of vascular and heart diseases, which were fatal in a considerable number of cases (WHO 2017). Subsequent studies, published in over 500 scientific publications, confirmed Nylander's conclusions.

To date, several different methods of lichen indication of air quality were developed and accepted. However, each of these methods has the same goal of precisely assessing the quality of the air; therefore all of the abovementioned methods show identical results. The lichen community approach is one of the widely used approaches.

Lichen communities are made up of a number of different lichen species and a whole range of biotic interactions that take place between them. The richness and number of species in lichen communities depend on the ecological conditions of the environment as well as the habitat itself. In altered habitat conditions, often caused by the presence of a pollutant, lichen communities may be composed of individual species, and such communities are called monospecific communities. The composition of a community also depends on the species' ability to respond to various conditions of the habitat (Parmesan and Yohe 2003; Root et al. 2003). While some species, which are distinguished by a pronounced presence and frequency even in altered environmental conditions, are characterized as tolerant and do not have any specific requirements, such as *Xanthoria parietina*, others are very rare and relatively poorly represented and therefore do not require specific environmental conditions.

Some lichens have limited dispersal abilities. The patterns of distribution, abundance, and interactions of species occur on different spatial scales and are influenced by ecological processes which also operate on multiple scales (Chase and Leibold 2002). There are two types of scales: local and regional. At the local scale, key factors for the composition of different lichen communities are niche differentiation, resource heterogeneity, and the interactions between competing species. On the other hand, the regional scale includes interactions of species that affect colonization and extinction dynamics among local communities.

Analyses of the lichen community show lichen responses to environmental factors (Koch et al. 2019; Aragon et al. 2019). The lichens shown react to environmental factors that operate on different spatial scales, and each factor has a particular range of influence, which is a consequence of different pollutant scattering patterns (Pinho et al. 2011). The composition of the lichen community depends on the variation of the microclimate. The microclimate is determined by the interactions between the regional climate, tree architecture, and bark properties. Furthermore, if we study the epiphytic lichen communities, we can notice that the bark of the tree, where the lichens can be found, has a major impact on the composition of the communities. The bark on the upper part of the trunk is generally smoother than the bark on the base of the tree. Most studies show that the pH value of the bark is a very important factor which affects the composition of lichen communities (van Herk 2001). Gombert et al. (2004) found that higher IAP (index of atmospheric purity) values were associated with nitrophytic and neutrophytic species, while sites with lower IAP values showed a predominance of acidophytic or oligotrophic species.

Lichen communities under the influence of the presence of various pollutants in the atmosphere are subject to various alterations in the structure of the communities themselves. Several studies have proven that the most sensitive lichen species completely disappear in urban and highly industrialized areas where the concentration of pollutants is the highest. In contrast, species tolerant of pollution survive even in moderately polluted areas (Davies et al. 2007; Larsen et al. 2007). Negative effects of pollutants on sensitive lichen species are manifested in inhibition and reduction of photosynthesis, respiration, chlorophyll content, membrane damage, and oxidative stress (Oztetik and Cicek 2011; Paoli et al. 2011; Majumder et al. 2013; Karakoti et al. 2014). Spatial organization of lichens' communities depends on many different factors, which act individually or complement each other.

Another approach used in studies of lichen indications of air quality is the *index of poleotolerance* (IP) (Trass 1973). This index describes the tolerance of lichen species to the presence of different air pollutants, which is measured on the basis of the level of coverage of each species individually as well as the total coverage of all species whose presence is detected at a given point of research.

This index can be calculated by the following equation:

$$\mathrm{IP} = \sum_{1}^{n} \left(\frac{a_i \times c_i}{C_i} \right) \tag{4.1}$$

where n is the number of species, a_i is the specific factor for each species which expresses tolerance to pollutants, c_i is the level of cover for each species, and C_i is the level of cover for all species at the station.

Values of IP also can be used for air quality assessment because their relation to IAP is proved (Martin and Martin 1974).

The *lichen diversity* (lichen diversity values, LDV) method is a widely used method in modern research on air quality bioindication. This approach represents the standard method of assessing the diversity of lichens on a substrate rich in organic substances, i.e., bark of different vascular plants. The basis for the development of this method is the norm – the rule that the size (complexity and development) of lichen diversity is directly proportional to air quality. In other words, as air quality decreases and pollution increases, the diversity of lichens also decreases. The results of the calculation of the LDV index are presented on the maps in the form of indication zones. The approach is based on the German and Italian models with several modifications (German VDI Lichen Mapping Guideline (VDI, 1995) and the Italian guideline (Nimis et al. 2002)). The main modifications concern several elements of subjectivity in the sampling process which were present both in the VDI and in the Italian guidelines. An abridged version of this guideline was published by Asta et al. (2002). The method proposed here determines the actual state of lichen diversity before or after long-term exposure to air pollution and/or to other types of environmental stress. The interpretation of geographic patterns and temporal trends of lichen diversity in terms of pollution, eutrophication, climatic change, etc. may be assisted by using ecological indicator values (e.g., Nimis and Martellos 2001, 2003) and a numerical analysis of a matrix of species (Gombert et al. 2004; van Haluwyn and Lerond 1988).

4.3 Method for the Calculation of the Value of Atmospheric Purity

One of the most commonly used methods of using lichen as a bioindicator for the biological indication of air quality is the *index of atmospheric* purity *(IAP)*.

This method involves a quantitative approach to air quality assessment that combines the number, frequency, and coverage of each lichen species in a particular study area (Kricke and Loppi 2002).

There are 20 different formulas for calculating the IAP (Conti and Cecchetti 2001). One of the most commonly used formulas for calculating the index of atmospheric purity is:

$$\mathrm{IAP} = \frac{1}{10}\sum_{1}^{n}\left(Q \times f\right) \tag{4.2}$$

where n is the number of lichen species found at a given station*,* Q is factor for accompanying species, and f is cover and frequency of each species.

The value for $Q \times f$ is divided by 10 to give a more manageable number (Johnsen and Søchting 1973).

Factor Q is calculated as follows:

$$Q = \frac{\sum_{j=1}^{m}\sum_{i=1}^{n} S_{ij}}{m} \tag{4.3}$$

where n is the number of species, m is the number of stations where the species of interest are present, and S_{ij} equals 1 if species i is present at station j (De Sloover and LeBlanc 1968).

This formula is still widely used in recent air quality assessments based on the calculation of IAP values, which is carried out in many research papers (Jayalal et al. 2017; Das et al. 2013; Attanayaka and Wijeyaratne 2013; Biazrov 2002). However, it has been shown that a simple method can very easily predict pollution levels with a certainty of over 97% (Gottardini et al. 1999).

Over time, many modifications involving various parameters like vitality and damage were developed. IAP values can be calculated using different equations:

$$\mathrm{IAP} = \sum_{1}^{n} Q \times C \tag{4.4}$$

$$\mathrm{IAP} = \sum_{1}^{n} Q \times C \times F \tag{4.5}$$

$$\mathrm{IAP} = \sum_{1}^{n} \frac{Q \times C \times F}{V \times S} \tag{4.6}$$

$$\mathrm{IAP} = \sum_{1}^{n} C \tag{4.7}$$

$$\mathrm{IAP} = \sum_{1}^{n} \frac{C \times F}{V \times S} \tag{4.8}$$

$$\mathrm{IAP} = \sum_{1}^{n} \frac{Q \times C}{V \times S} \tag{4.9}$$

$$\mathrm{IAP} = \sum_{1}^{n} F \tag{4.10}$$

$$\mathrm{IAP} = \sum_{1}^{n} Q \times F \tag{4.11}$$

$$\mathrm{IAP} = \sum_{1}^{n} Q \tag{4.12}$$

where Q is the factor of accompanying species, C is the cover scaled from 0 to 5, F is frequency value scaled from 1 to 10, V is vitality (three levels, very good, moderate, poorly developed), and S is damage (three levels, no, moderate, strong damage).

IAP calculation formulas are able to predict the levels of eight air pollutants measured by automatic control stations. Swiss authors (Amman et al. 1987; Herzig et al. 1989; Herzig and Urech 1991) tested all 20 different IAP formulas, comparing IAP values with direct measurements of eight air pollutants (SO_2, NO_2, Pb, Cu, Cd, Zn, Cl, and dust), and found the best correlation with the formula:

$$\mathrm{IAF} = \sum_{1}^{n} f \tag{4.13}$$

where f is the lichen cover.

Many scientists from Germany, Spain, Slovenia, and Japan have proposed modifications to the original formula for locally calculating IAP values.

The correlation between air quality and abundance of epiphytic lichens is well known and has been widely used in the assessment of air quality (Nimis et al. 2002). Although IAP values are often interpreted as indicators of air pollution, differences in IAP or lichen species richness can also arise due to a number of habitat conditions (Gombert et al. 2004; Perhans et al. 2009).

In this research, the above formula was used as a simple and reliable model for calculating the value of the atmospheric purity index. The aim of the research is to observe the differences between the air quality of one anthropogenic – urban – and one natural or seminatural ecosystem based on the variation of IAP values presented through two case studies, the town of Kuršumlija (case study 1) and Prolom Banja (case study 2).

4.3.1 Calculation of the Index of Atmospheric Purity

With the help of working ladders (sample ladder) measuring 50 × 10 cm, the lichen cover is determined by a numerical method. The grid is placed on each substrate unit (Fig. 4.1).

When researching epiphytic lichens, only trees at a height of 1–1.5 m from the ground are considered, whose angle of inclination does not exceed 5°. The number of the working network squares in which the presence of each species is observed is expressed as a percentage for each substrate unit. Since each point of research consists of several (5–10) close substrate units (trees), the arithmetic mean gives the values of coverage in percentages of each species individually. The *f* coefficient is expressed in units (1–5) and represents the degree of coverage of each type of lichen on a particular substrate. It is determined on the Braun-Blanquet model scale, a method applied in research of higher plant communities.

The cover of each ascertained lichen species is expressed by values from 0 to 10, on a scale which characterizes the cover: values of 9–10 are assigned to species with a very high frequency of being found and a very high degree of cover (from 80% to 100%); values of 7–8 are assigned to species with a high frequency of being found or a high degree of cover (60–80%); values of 5–6 are assigned to species that are not found often or that have a low degree of coverage (from 40% to 60%); values of 3–4 are assigned to species that are rare or have a low degree of cover (from 20% to 40%); and values of 0–2 are assigned to species that are very rare and with a very low degree of cover (individually or from 0% to 20%) (Biazrov 2002).

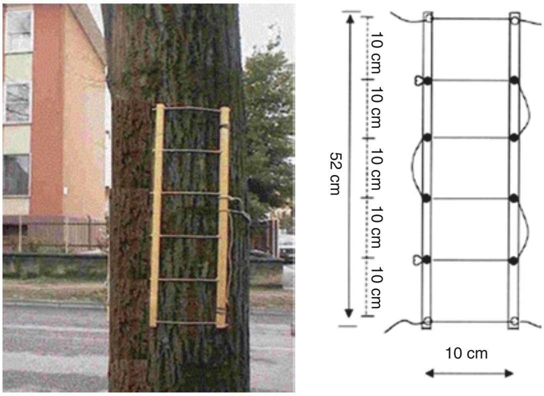


Fig. 4.1 Example of a sampling ladder

In the field notebook, next to each point of research, the type of substrate on which lichens were found is recorded, as well as the values of the taxon cover coefficient at each point of research. The frequency of each type is calculated in relation to the percentage representation on the total number of investigated points. The ordinal numbers of the investigated points and their coordinates are entered on the working map of the investigated area.

The studies mentioned here (case study 1 and case study 2) use the numerical method of calculating the index of atmospheric purity. According to Loppi et al. (1997) and Kricke and Loppi (2002), IAP (*i*ndex of *a*tmospheric *p*urity) values are calculated according to the formula:

$$\mathrm{IAP} = \sum f \tag{4.14}$$

where f represents the lichen cover.

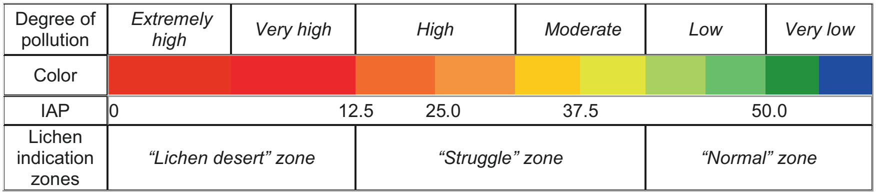
The index of atmospheric purity is calculated for each point of research within the research area. The air quality assessment scale (Conti and Cecchetti 2001) is used to assess the degree of air pollution and to determine the lichens' indication zones. Higher index values indicate better air quality, while lower values indicate that the quality of the air is lower (Table 4.1).

Based on the IAP values, three basic air quality zones can be distinguished:

1. The "normal" zone occupies areas where the air is clean or there is no significant air pollution ($\mathrm{IAP} > 37.5$).
2. The "struggle" zone covers an area with a moderate level of pollution ($12.5 \le \mathrm{IAP} \le 37.5$).
3. The "lichen desert" zone occupies the territory with the highest degree of air pollution ($0 < \mathrm{IAP} \le 12.5$) (Conti and Cecchetti 2001).

In order to obtain a more complete and clearer picture of the state of air quality based on lichen monitoring, the IHI method is also used, i.e., IAP values are often supplemented in modern research by calculating the values of the anthropogenic impact index (IHI) (Gombert et al. 2004). By calculating the index of anthropogenic impact, an approximate picture of the quality and quantity of the impact of various human activities on the observed area was obtained, i.e., ecosystem or wider landscape. The IHI method is a complementary method used in research of this type for the comparative assessment of air quality and anthropogenic impact. The values of

Table 4.1 Air quality assessment scale based on pollution levels and IAP values (Conti and Cecchetti 2001)



Degree of pollution	*Extremely high*	*Very high*	*High*	*Moderate*	*Low*	*Very low*
Color	🟥	🟥	🟧🟧	🟨🟨	🟩🟩	🟩🟦
IAP	0	12.5	25.0	37.5		50.0
Lichen indication zones	*"Lichen desert" zone*		*"Struggle" zone*		*"Normal" zone*	

the IHI index are often inversely proportional to the IAP values, so the points of research where the IHI values are high are often characterized by low IAP values and vice versa. Values of index of anthropogenic impact (*i*ndex of *h*uman *i*mpact, IHI) are calculated for each point of research individually. Natural areas which are undisturbed ecosystems like protected natural assets, where it is not necessary to calculate IHI values, may be exceptions. Gombert et al. (2004) indicate that IHI values aim to characterize each point of research using the following environmental parameters: degree of urbanization (U, *U*rbanization), traffic intensity (T, *T*raffic), local development (D, Local *d*evelopments), and exposure (E, *E*xposure). Categories 1 to 4 are assigned to each parameter to represent the gradient of alternations.

4.4 Study Area and Sampling Procedure

The research of air quality indication is conducted by field work which includes the collection of data on the presence and frequency of lichens at predefined points of research. The number of investigated points varies depending on the size, type, and conditions of the area under investigation. Each investigated point consists of several substrate units, usually five. Substrate units consist of an organic base, i.e., the bark of various plant species in forests, tree lines, parks, orchards, and other green areas. The research includes the analysis of the presence and frequency of epiphytic (lignicolous and corticolous) lichens.

The method of lichen indication of air quality using lichen as a bioindicator first involves the identification of lichen, usually done on the spot, and only small parts of the thallus of those types of lichen that require more detailed tests and whose identification could not be done on the spot are collected. Numerous and different determinants in the form of textual guides are available for the identification of lichens, which are based on the dichotomy of the appropriate or inappropriate expression, the so-called identification keys like Wirth (1995) and Dobson (2005).

Appropriate tools (e.g., a knife or a geological hammer) are used to collect samples in the field, since the collected lichen samples are found on the bark of plant species and are sometimes firmly attached to the substrate. The standard procedure further assumes that the samples are packed in appropriate packaging, on which ordinal numbers are recorded. These same ordinal numbers are entered into the field diary as all other relevant data.

Determining the important, morphological and anatomical, character of lichens is done with the help of a laboratory magnifying glass or light microscope. For the determination of different types of lichens, it is sometimes necessary to treat the bark or the marrow of two (by all characteristics) similar specimens of lichens with certain chemical substances – reagents – in order to determine (or make) (according to the entries from the determinant) the difference between the analyzed, similar specimens, on the basis of a qualitative or quantitative difference in the reaction to the reagent used. Dyed chemical reactions with the following compounds –

reagents – are most commonly used: $NaClO_2$, KOH (50% solution), P (paraphenylenediamine) reagent, and Lugol's solution.

4.4.1 Kuršumlija Town Case Study: Geoclimatic Features

The town of Kuršumlija is located in the Toplica District, which covers an area of 2231 km^2 and belongs to the south of the Republic of Serbia (Fig. 4.2). The territory of Serbia is located in the central part of the Balkan Peninsula. The geographical position of the Toplica District is determined by the coordinates 42°54′–43°24′ north latitude and 18°30′–19°30′ east longitudes.

The town of Kuršumlija is an urban area of 952 km^2 with 13,306 inhabitants (SORS 2011). This urban area is located at an altitude of 383 m (above the sea level). The climate is moderately continental. The most frequent wind blows from the southwest 310‰, followed by north 171‰ and the northeast 162‰. The "wind rose" in Kuršumlija area provides a graphical representation of these data (Fig. 4.3). The mean annual temperature is 11.6 °C. The air in the researched areas of the municipality of Kuršumlija is moderately humid throughout the year, because the average annual relative humidity is 73%. The mean annual rainfall is 968.3 mm, which is much higher than in the surrounding towns (RHSS 2019).

Kuršumlija town used to have a developed textile, wood, and metal industry, which were sources of air pollution. Nowadays, the aforementioned industries work with reduced capacity and with a relatively developed motor traffic, and individual household fireplaces are the main sources of the decrease in air quality. Bearing in mind that there are no measuring stations for the physicochemical monitoring of air quality in Kuršumlija, all previous investigations of air quality were performed using the biological monitoring method.

Fig. 4.2 Geographical position and satellite image of the town of Kuršumlija

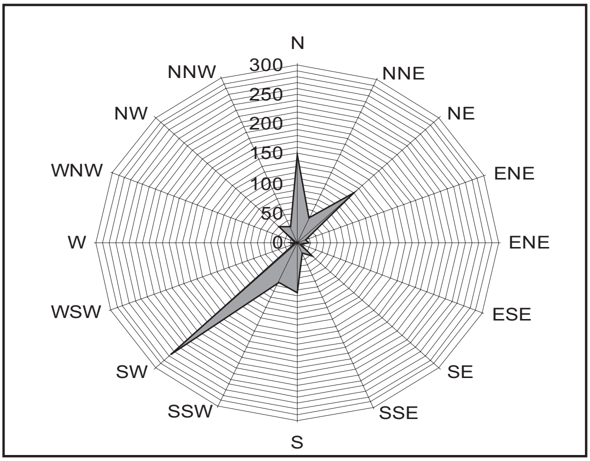


Fig. 4.3 "Wind rose" in the town of Kuršumlija (The Republic Hydrometeorological Institute of Serbia)

4.4.2 *Prolom Spa Case Study: Geoclimatic Features*

Prolom Spa is located in the submountain or subalpine climate, 23 km southeast of Kuršumlija (Fig. 4.4). This spa center is located at a height of 598 m and is framed by the Sokolovica mountain (1050 m) and Arbanaska mountain (1.009 m) in the north, followed by the peaks of the Radan mountain-Samar (1008 m), Vijogor (1128 m), and Sokolovac (1260 m), in the east, and Prolomska mountain (1104 m) and Mejanska mountain (962 m), in the south, while in the west, along the valley of the Prolomska river, it is somewhat more open toward the valley of Kosanica. Long

Fig. 4.4 Satellite image of the Prolom Spa investigated area

and cold winters (55–80 days) characterize the climate of Prolom Spa, while summers are shorter (less than 60 days) and chillier. This climate is suitable for the recovery of patients, so this spa settlement, thanks to its geographical position as well as the healing properties of the water from numerous springs, attracts an increasing number of tourists every year. The settlement area covers an area of 18.10 km^2, making it one of the medium-sized settlements in the municipality (Archive of the TO Kursumlija). Today, 131 people live in Prolom Spa. In the following decades, the number of tourists slowly increased. Nowadays, Prolom Spa stands out among spa tourist centers of national importance in terms of development perspective.

Prolom Spa is located on the southern slopes of Radan mountain. Although the forests in the wider area of Prolom Spa are largely preserved, different types of secondary vegetation still predominate in this area, most often mountain meadows and pastures. In addition, valley meadows, rock vegetation, and clearing vegetation also appear in the researched area. The associations that develop on bare andesitic rocks and in their cracks are certainly of great biodiversity importance.

This seminatural ecosystem does not have large air pollutants, given that the anthropogenic impact is relatively small and is reflected in the fact that the heat of thermal water sources is widely used for heating buildings instead of individual furnaces, which further reduces the emission of harmful gases.

Prolom Spa is one of the most attractive spa and rehabilitation centers visited by a large number of tourists. In addition to regional exports, the specialized factory for filling and transport of the “Prolom Voda” water with healing properties “Planinka” also transports and exports water to the different countries. The increased intensity of individual, public, and motor traffic in order to meet not only the daily needs of citizens and tourists but also the realization of economic activities certainly has an impact on air quality. The air quality of this ecosystem is also affected by the increasingly intensive construction of facilities to increase the accommodation capacity of tourists.

4.5 Results and Discussion

4.5.1 Results from Kuršumlija Town Case Study

In the urban ecosystem of Kuršumlija, the presence of 42 species of lichens from 23 genera was recorded at 15 investigated points (Table 4.2). Epiphytic species were the most numerous. Out of a total of 40 epiphytic species, 25 have the foliose type of thallus, 12 have the corticolous type, and only 3 species have the fruticose type of thallus. The presence of two saxicolous lichen species was also noted. The most frequent species (100%) were *Hypogymnia physodes* and *Physcia adscendens* whose presence was constant at all points of research. *Parmelia sulcata* and *Xanthoria parietina* had a high frequency (93.33%). Among the most common are

Table 4.2 Values of IAP and IHI for each investigated point in Kuršumlija

Number of investigated points	Coordinates	IAP values	IHI values
1	43° 8′43.00″N 21°15′55.57″E	28	18
2	43° 8′23.61″N 21°16′2.47″E	23	24
3	43° 8′29.30″N 21°16′20.71″E	10	36
4	43° 8′23.10″N 21°16′18.40″E	11	32
5	43° 8′13.77″N 21°16′6.30″E	24	24
6	43° 8′11.40″N 21°16′32.00″E	21	21
7	43° 8′25.69″N 21°16′42.92″E	14	36
8	43° 8′19.10″N 21°16′50.34″E	16	28
9	43° 8′26.15″N 21°16′50.74″E	18	28
10	43° 8′31.70″N 21°17′9.50″E	22	21
11	43° 8′38.10″N 21°16′49.40″E	22	21
12	43° 8′47.10″N 21°16′37.40″E	24	15
13	43° 8′35.82″N 21°17′45.43″E	26	15
14	43° 8′23.64″N 21°17′32.96″E	21	18
15	43° 8′14.12″N 21°17′7.46″E	22	18

species with a foliose type of thallus. The species *Lepraria aeruginosa* and *Xanthoparmelia somloënsis* had the lowest frequency (6.67%).

The species of vascular plants on which lichens were found were mostly *Acer campestre* L., *Cornus mas* L., *Juglans regia* L., *Malus domestica* Borkh., *Robinia pseudoacacia* L., and *Tilia platyphyllos* Scop.

By processing the obtained data, IAP values were calculated, which varied from 10 to 28. IHI values ranged from 15, as calculated in points 12 and 13, to 36 as calculated in investigated points 3 and 7 (Table 4.2). The air quality of this urban ecosystem was analyzed for the first time using the biological-lichenological indication of air quality in a paper published in 2019 (Ristić et al. 2019).

4.5.2 Results from Prolom Spa Case Study

In the area of Prolom Spa, the presence of 72 species of lichens from 33 genera (Table 4.3) was recorded at 15 investigated points, of which 57 were species of epiphytic lichens, 20 species with a corticolous type thallus, 31 species with a foliose type thallus, and 6 species with a fruticose type thallus. The presence of ten species of terricolous and five species of saxicolous lichens was also noted. The most frequent species (100%) were *Hypogymnia physodes*, *Lecidella elaeochroma*, *Parmelia sulcata*, *Phaeophyscia orbicularis*, *Physcia adscendens*, and *P. stellaris*, whose presence was noted on all investigated points. The following species also had an extremely high frequency (93.33%): *Flavoparmelia caperata*, *Hypogymnia tubulosa*, *Melanelixia subaurifera*, and *Xanthoria parietina*. The presence of certain

Table 4.3 Values of IAP and IHI for each point of research in Prolom Spa

Number of investigated points	Coordinates	IAP values	IHI values
1	43° 2′53.61″N 21°24′6.51″E	50	14
2	43° 2′49.75″N 21°23′52.31″E	48	10
3	43° 2′46.31″N 21°23′58.76″E	43	24
4	43° 2′47.93″N 21°23′50.46″E	52	14
5	43° 2′46.56″N 21°24′14.52″E	53	12
6	43° 2′45.71″N 21°24′10.92″E	55	12
7	43° 2′52.18″N 21°23′59.22″E	45	12
8	43° 2′42.35″N 21°24′0.71″E	56	14
9	43° 2′43.36″N 21°23′58.37″E	43	8
10	43° 2′43.57″N 21°24′5.80″E	45	16
11	43° 2′51.40″N 21°23′54.87″E	45	16
12	43° 2′39.82″N 21°23′57.24″E	49	18
13	43° 2′43.54″N 21°23′52.64″E	45	14
14	43° 2′46.77″N 21°24′2.45″E	40	14
15	43° 2′50.16″N 21°24′1.88″E	43	18

species that have an extremely low frequency (6.67%) was also confirmed in the area of investigation: *Lecanora confusa*, *L. glabrata*, *M. subargentifera*, *Parmelia conspersa*, *Parmelina quercina*, *Physconia detersa*, *P. distorta*, *Rhizocarpon geographicum*, and *Rinodina colobina*.

The substrate on which the presence of lichens was recorded, and from which samples of individual species were collected, was most often the bark of plant species: *Quercus frainetto* Ten., *Q. cerris* L., *Prunus domestica* L., *Robinia pseudoacacia* L., and *Juglans regia* L.

For the study area, the range of variation of IAP values ranged from 40 to 56. IHI values ranged from 8 to 24. The lowest value was calculated at point 9, while the highest value was calculated at point 3 (Table 4.3).

Different investigated points ascertained the presence of different numbers and composition of taxa; therefore the variation of IAP values was noticed accordingly. IHI values at different investigated points also differed depending on the values of general environmental parameters: degree of urbanization, traffic intensity, local development, and exposure.

Based on the results of variation of IAP and IHI values at different investigated points, it is noticeable that these values are inversely proportional. The investigated points at which high IAP values were calculated were characterized by low IHI values and vice versa. The results clearly show that the highest IHI values were recorded in the investigated area of Kuršumlija. The calculated IAP values were also the lowest in this area.

The air quality of this ecosystem was analyzed for the first time using the biological-lichenological indication of air quality in the papers in 2020 (Ristić et al. 2020; Djekić et al. 2020).

4.5.3 Mapping the Air Quality Indication Zone

To determine the zones of lichen indication of air pollution, science has developed very sophisticated methods with statistical calculations, as well as appropriate computer programs, all with the aim of avoiding subjectivity while determining zones of different degrees of air pollution. At the same time, their consistent application makes it possible to compare research in different and, at the same time, very distant areas.

By arranging the points of research on the basis of the IAP value on the map of the research area, the contours of the boundaries of the lichen indication zones of different degrees of air quality of the investigated area are obtained. In order to more clearly and precisely visualize the obtained indication zones, various tools of geographic information systems are used.

Geographic information systems are successfully applied in various ecological studies with the aim of detecting, identifying, describing, and monitoring the state of the environment in both aquatic and terrestrial ecosystems in a modern and reliable way. This is a very useful tool in estimating the spatial distribution of air pollution. Many authors point out the value of implementing the GIS application in ecological studies of this type (Sohrabinia and Khorshiddoust 2007; Ulshöfer and Rosner 2001; Gerdol et al. 2014). The interpolation method of universal kriging with a linear variogram (Davis 1986) is one of the most reliable methods used to construct charts showing the formed air quality indication zones based on IAP values. For the graphical representation of the distribution of points with different IAP values, percentile charts (scale divisions) are used where different colors show different IAP values.

4.5.3.1 Case Study: Integral View

The general chart of air quality indications shows all research areas with the aim of visually comparing the air quality conditions in different areas. The presence of three basic zones of air quality indication is noticed – "normal" zone, "struggle" zone, and the "lichen desert" zone (Fig. 4.5).

The lichen "normal" zone is shown in different shades of lighter green and dark green-blue, based on a scale with IAP values (Fig. 4.5). It is characterized by low or very low pollution. This zone is characterized by values of IAP >37.5 (Table 4.1). The "normal" zone is present in the entire research area of Prolom Spa.

The lichen "struggle" zone is shown in different shades of orange and orange-yellow (Fig. 4.5). It is characterized by a high or moderate degree of pollution (Table 4.1). This zone is characterized by the values of 12.5 ≤ IAP ≤ 37.5 and

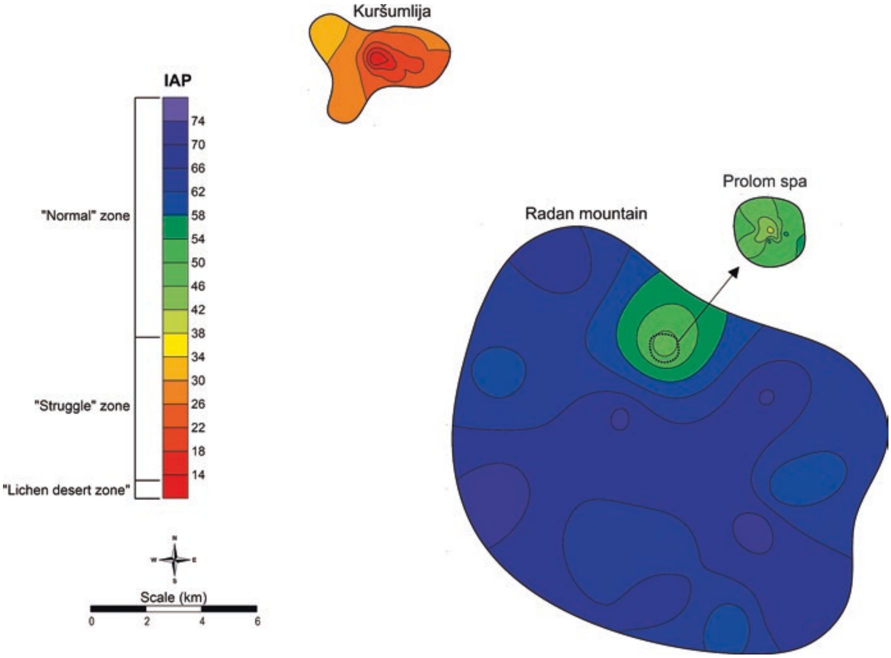


Fig. 4.5 Spatial distribution of different zones to indicate the air quality for both investigated areas (Case studies)

covered almost the entire investigated area of the town of Kuršumlija. The map of the air quality indication shows the “struggle” zone in Kuršumlija including peripheral parts of the city marked with shades of light orange – a smaller, mostly peripheral part of the study area where the research points are characterized by values of $25 \leq \text{IAP} \leq 37.5$ – and dark orange, most of the surface of the area where the points of research are located, whose values are $12.5 \leq \text{IAP} \leq 25$.

The zone of the “lichen desert” on the general chart is shown in shades of distinct red (Fig. 4.5). According to the scale of air quality assessment, the “lichen desert” zone is characterized by extremely high and very high air pollution (Table 4.1). IAP values indicating an extremely high degree of pollution were not obtained in this paper. The zone of the “lichen desert” is characterized by the values of $0 < \text{IAP} \leq 12.5$. This zone is present in the investigated area of the center of Kuršumlija.

4.5.3.2 Kuršumlija Case Study (Case 1)

On the map of the town of Kuršumlija, we notice the presence of two basic zones of lichen indication (Fig. 4.6). The lichen “struggle” zone is located in the larger part of the investigated area of the town of Kuršumlija, while the “lichen desert” zone covers another, smaller part of the area. The picture of the distribution of lichen zones of air quality indication in the investigated area of the town of Kuršumlija shows that the basic zones are not monolithic, but there is a division into subzones in this area. The “struggle” zone (B) in the investigated area of the town of Kuršumlija is divided into two subzones. The first subzone or “outer struggle zone” (B_1) covers the northeastern and northwestern part of the urban investigated area. Investigated points 1, 5, 12, and 13 are located in this subzone. The IAP values of these points range from 24 to 28, which are the highest IAP values in relation to other points of the town investigated area. The second formed subzone or “the inner struggle zone” (B_2) continues to the “lichen desert” zone and extends to the southeastern part of the city area. It includes nine investigated points (2, 6, 7, 8, 9, 10, 11, 14, and 15). The IAP values range from 14 (point 7) to 23 (point 2).

The zone of “lichen desert” (C) covers the center of the town and is characterized by the presence of only two points of research – 3 and 4. This zone is characterized by very low IAP values – 10 and 11. This zone occupies a small part of the area, primarily the socially active town center. This is expected because it is under a more intense anthropogenic influence. However, this zone shows tendencies toward a possible transition to a “struggle” zone, because the presence of species that are moderately tolerant to air pollution, such as *Lecidella elaeochroma*, has been recorded at the mentioned points. The “struggle” zone includes points which had higher IAP values, so the conclusion is that this part of the area is characterized by a higher degree of air quality.

The biggest problem when it comes to air quality in Kuršumlija is the pollution incurred by the “Kopaonik” wood industry where fuel oil and wood waste are used for heating work rooms and for the steam boiler needs in the production process. The combustion of these substances constantly emits pollutants, with a large amount of inert dust. In addition to “Kopaonik,” boiler rooms of other work organizations

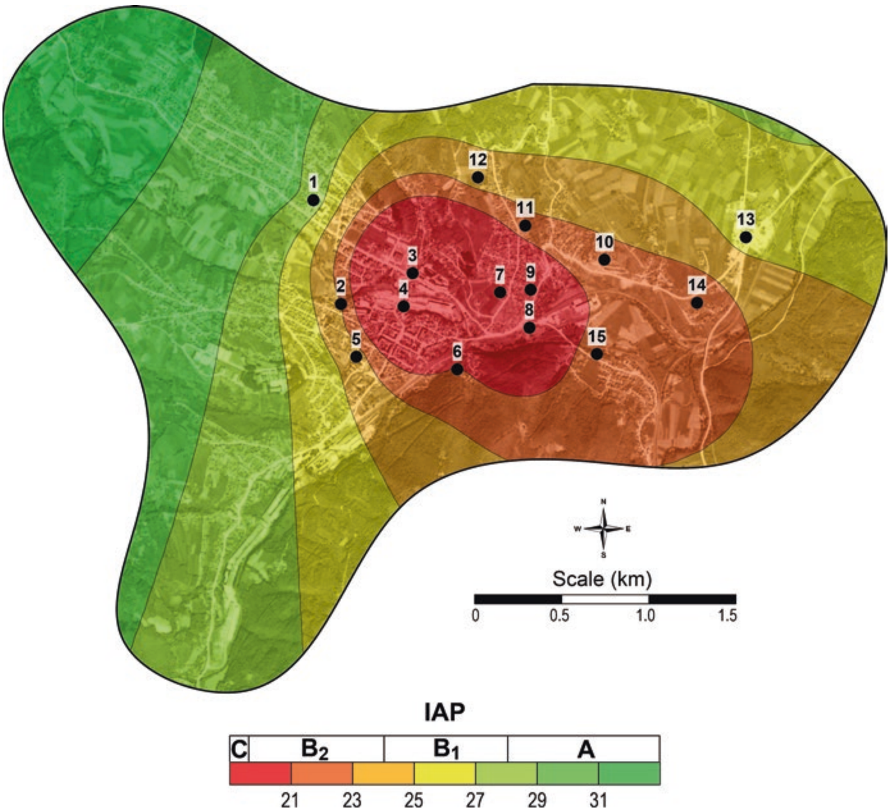


Fig. 4.6 The spatial distribution of the different indication zones of air quality in Kuršumlija (A, lichen "normal" zone; B, "struggle" zone (B_1 "outer struggle" zone and B_2 "inner struggle" zone); and C, "lichen desert" zone)

and institutions located mainly in the town center also appear as potential sources of air pollution in the town. Household fireplaces which use wood and coal as firewood are also present during the winter months. Car exhaust gases are also air pollutants, because the bulk of the atmospheric releases of nitrogen oxides, carbon monoxides, hydrocarbons, and lead compounds originate from cars.

4.5.3.3 Prolom Spa Case Study (Case 2)

The investigated area of Prolom Spa is located in the lichen "normal" zone of air quality indication (Fig. 4.7). A more detailed presentation of the investigated area shows the division of the "normal" zone into three subzones – A_1, A_2, and A_3. The first subzone (A_1) is characterized by points 3, 7, 9, 10, 11, 13, 14, and 15 which are distinguished by the lowest values of IAP (from 40 to 45) in relation to other investigated points in this area. The second subzone (A_2) is characterized by points 1, 2,

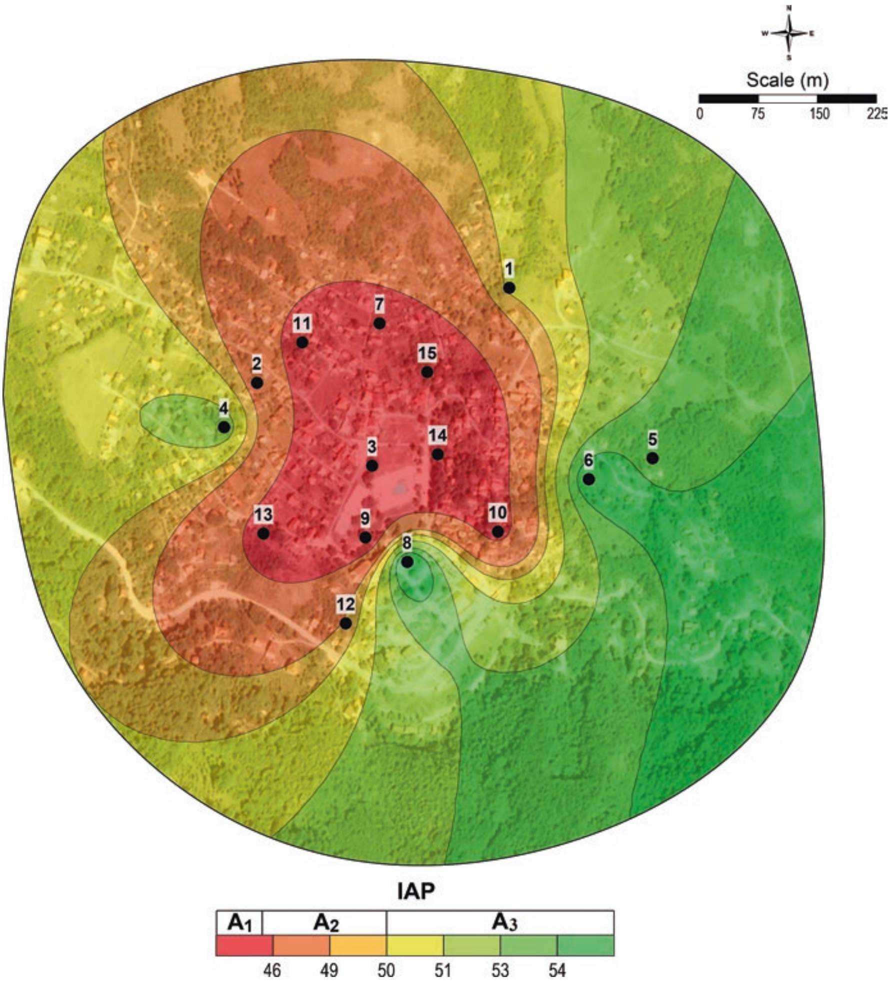


Fig. 4.7 Map of the spatial distribution of different zones of air quality in Prolom Spa (A_1, A_2, and A_3 subzones of the "normal" zone)

and 12, whose IAP values vary from 48 to 50. The third formed subzone (A_3) is characterized by five investigated points – 4, 5, 6, 8, and 52. It mainly covers the southeastern part of the area and is of a discontinuous shape, so its fragments are located in the northwest (point 4). The IAP values of the points located in this subzone vary from 52 to 56.

It should be borne in mind that the zones of lichen indication of different degrees of air quality are a reflection of its condition in a particular area over a long period of time (Mayer et al. 2013; Sujetoviene 2015).

Slaby and Lisowska (2012) recorded identical results within their research conducted in Krakow. The subject of their research was the recolonization of epiphytic lichens in the urban ecosystem as a consequence of air quality improvement. In addition, Duque et al. (2016), as part of their research, present the evaluations of strategies for reducing air pollution in urban ecosystems. According to Llop et al. (2012), traffic intensity, green areas, and individual household fireplaces have a great influence on air quality and the reduction of lichen diversity. Previously conducted research had shown identical results (Conti and Cecchetti 2001; Loppi 2014; Washburn and Cullen 2006; Perlmutter 2010; Sujetoviene 2015; Sujetoviene and Galinyte 2016).

4.5.4 Statistical Analyses

The statistical analysis of data obtained by field research can be performed with the help of several widely accepted static tools. The application of artificial neural networks has proven to be very effective in determining the spatial distribution patterns of lichens. In our previous research, Kohonen's self-organizing maps were used (*s*elf-*o*rganizing *m*aps, SOM; Kohonen 1982, 2001), which represent a type of artificial neural network, i.e., an unsupervised algorithm (Ristić et al. 2019; Ristić et al. 2020). The SOM method is a technique of modeling and visualization of linear and nonlinear connections in a high-dimensional data set in the form of a low-dimensional space (neural network). Self-organizing maps differ from other types of neural networks in that they store information about the topological properties of inputs using the function of neighboring neurons. The Kohonen network consists of a number of neurons, each of which contains a set of weight factors of equal length as the input data vector. The structure of the SOM consists of an input and an output layer.

The data is entered into the analysis using an input matrix. The input matrix for displaying the spatial distribution of lichens consists of a certain number of rows and columns. The number of rows represents the number of investigated points, while the number of columns represents the number of ascertained taxa. A self-organizing map can be interpreted as a set of neurons, each of which represents one group of data, with the data belonging to the group represented by the "winning" neuron. Upon completion of network training, all localities were assigned to the appropriate neurons. The resolution of the SOM network depends on the type of analysis.

As the SOM analysis does not show statistical indications of the species responsible for cluster separation, this type of analysis is often accompanied by the application of the IndVal method (the *ind*icator *val*ues; Dufrêne and Legendre 1997). In order to identify the significant species with IndVal parameter values greater than 25%, the Monte Carlo significance test with 1000 permutations is applied. Such species are representative of the group of localities with a relative frequency and density of no less than 50%. Species that have an IndVal value of less than 25%, and are statistically significant ($p < 0.05$), represent taxa that are important for the group. These species provide additional information about the group but are not representative because they have low relative frequency values. The IndVal method analysis can be performed using the PC-ORD 4.0 software package (McCune and Mefford 1999).

Statistical analysis of data obtained by lichen indication of air quality can also be obtained by using statistical software packages, SPSS 19.0 (SPSS Science, Chicago, USA), while Origin 9.0 (OriginLab Corporation, Massachusetts, USA) (Xu et al. 2018) is often used for charting and regression analysis.

4.6 Conclusion

Air quality is an important topic nowadays. Intensified urbanization and industrialization in the twenty-first century have led to the deterioration in air quality, which has certainly affected human health. The number of patients with respiratory diseases, who in some cases had had a fatal outcome, has sharply risen. Therefore, there is a need for a timely detection of air pollution levels and tracking changes. Research on the biological indication of air quality is one of the powerful tools for monitoring air quality, which aims to lead to the improvement of air quality and, thus, to the improvement of human living conditions. Methods of biological indication include the use of indicator organisms, and lichens have proven themselves to be very good indicator organisms of air quality. One of the more convenient and reliable methods of lichen indication of air quality is the method of calculating the value of the atmospheric purity index. Based on the mentioned values, a complete picture of the air quality of an area, the spatial distribution of pollution, and the types that can be indicators of air quality is formed. It is this exact method that has been applied in the two case studies presented in this paper.

Based on this research, it can be concluded that the investigated area of the town of Kuršumlija is characterized by relatively poor air quality. It is an urban area with relatively well-developed motor traffic, and in addition, a large amount of harmful substances is emitted daily from the exhaust openings of the wood industry, which negatively affect air quality and the environment in general. The state of air quality is also affected by individual household fireplaces. In a large number of cases, wood and coal are used as basic raw materials for heating, the combustion of which releases oxides of sulfur and carbon. The reason for this threat to air quality is reflected in the poor distribution of industrial, residential, and other socially active zones in relation to the prevailing geoclimatic factors. In the end, the lack of public greenery in this urban settlement, which obviously cannot be compensated by sporadic greenery from the yards of family homes and city parks, is also one of the indirect causes of reduced air quality.

The second case study, in Prolom Spa, where the air quality is at a high level, painted a completely different picture. The obtained results of air quality research in this area indicate the presence of an exclusively lichen “normal” zone.

Based on all of the above, it is possible to conclude that lichens are good indicators of air quality and could, in certain cases (e.g., when there is no possibility of a physicochemical detection), be an excellent alternative but also a regular complementary method of air quality detection, especially due to the more favorable economic aspect of use.

4.7 Future Prospective

Bearing in mind the presented history of the use of different methods of lichen indication of air quality, future research in this area would lead to changes in order to develop and improve existing methodologies and software. More intensive scientific research and work will provide a more complete and objective picture of the state of air quality in the wider area of the Republic of Serbia and even Europe by applying a similar methodology. Future perspectives are directed toward intensified work on the formation of a new modernized scale of air quality assessment which, unlike the already existing scale of qualitative assessment of epiphytic lichen susceptibility for England and Wales (Hawksworth and Rose 1970), would be applied in a wider area. Based on previous research on air quality lichen monitoring, future research would combine several indicative methods of biomonitoring with physicochemical measurements of pollutant concentrations, which are the most common causes of air pollution, because synergy leads to clearer and more precise achievement of goals.

The World Health Organization (WHO) points out that countries working intensively to detect and reduce levels of air pollutants can have a significant impact on reducing the incidence of cardiovascular and respiratory diseases such as lung cancer (WHO 2018). European Air Quality Directive (EU 2008) defines the Data Quality Objective (DQO) that monitoring methods need to comply with to be used as indicative measurement for regulatory purposes, a large number of cities still do not have adequate measuring stations for automatic measurements of pollutant levels, so the application of biomonitoring in these areas is necessary as it is the only source of detecting the real state of air quality in a longer period of time.

Adequate procedures in industrial development and urban planning based on the results of environmental biomonitoring in the future should be imperative (conditio sine qua non).

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Chapter 5
Proposing Chemometric Tool for Efficacy Surface Dust Deposition Tracking in Moss Tissue Cross Bioindication Process of Metals in Environment

Biljana Balabanova, Maja Lazarova, Blažo Boev, Lucian Barbu-Tudoran, and Maria Suciu

Abstract Mosses have been used for biomonitoring in a number of different ways which may lead to rather different results, and some kind of classification seems necessary at this point. *Epigeic* mosses (growing on the ground) are preferred in the regional surveys in Europe. Trace elements may be either absorbed on the moss from the atmosphere as soluble chemical species in wet deposition or contained in particles from dry deposition. Part of the trace element content of particulates may eventually be released by weathering and reabsorbed by the moss. Whereas uptake efficiencies for particulate-bound trace elements are generally poorly known, ions may be subject to active uptake into cells or attached on the moss surface by physical and chemical forces. Methods are available to distinguish between intracellular and surface-bound fractions of elements. The main problem with the issue of moss biomonitoring is revealed as (a) the transport of soluble compounds from the soil into moss tissue, particularly during periods with excessive soil/water contact. Although mosses do not have a root system, influence from this source cannot be disregarded, in particular in areas with low atmospheric deposition and (b) wind-blown mineral dust from local soil. As far as the surface-bound fraction is concerned, little is known about the binding mechanisms, but the fact that different

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metals show rather large differences in their retention capacities indicates that both simple cation exchange on negative surface charges and complex formation with ligands on the moss surface are involved. Laboratory analysis using scanning electron microscopy has been involved for the determination of the dry deposition occurring within moss bioindicators.

Keywords Moss bioindication · *Homalothecium lutescens* · *Hypnum cupressiforme* · Toxic metals · Air deposition · SEM

5.1 Introduction in Anthropogenic and Non-anthropogenic Air Deposition's Impacts

Air pollution is a serious concern because limit values, especially those for suspended particulate matter concentrations set to protect human health, are being exceeded (Longchurst and Brebbia 2013). Several sources and causes of major air quality problems have been identified, but they may differ within urban and other environments (Beelen et al. 2009). The use of wood for heating households in the winter period causes serious problems with air quality in densely populated residential areas, as most households in the country still use wood as their primary source of heating. Road traffic is also a source of air pollution in urban areas, due to high traffic intensity and partly due to the old fleet and inadequate vehicle maintenance (De Miguel et al. 1999; Longchurst and Brebbia 2013). Energy production and industry can affect air quality locally, especially in the vicinity of old industrial plants that do not have modern emission reduction systems. An additional impact on air pollution is the development of densely built-up urban areas and the reduction of green areas (Finlayson-Pitts and Pitts 1999; Beelen et al. 2009; Longchurst and Brebbia 2013).

Inefficient combustion in household fireplaces and boilers, together with the use of poor-quality firewood, i.e., wet or processed wood, and even waste materials, causes problems, especially in densely populated residential areas. A significant proportion of households in the country still use firewood as their primary heat source. Outdated and limited central heating systems, as well as the high cost of electricity, further increase the use of solid fuels for household heating. Power generation and distribution systems are often outdated, inefficient, and unreliable and pollute the air (Finlayson-Pitts and Pitts 1999).

Electricity production mainly depends on poor-quality lignite and old thermal power plants. Combined heat and power generation, as well as wind and hydroelectric power generation, is still underrepresented in underdeveloped countries (Goodarzi 2006). Lack of proper waste management and recycling systems increases the amount of uncontrolled waste incineration, such as the incineration of household waste in the open. Also, burning agricultural waste can cause air quality problems locally.

The impact of road traffic emissions is greatest in urban areas with a congested road network and a high frequency of vehicles. Road traffic accounts for emissions of nitrogen oxides, carbon monoxide, benzene and suspended particles, heavy metals, and polycyclic aromatic hydrocarbons. Road traffic emissions depend on the type and age of the vehicles, the number of kilometers traveled by each group of vehicles, as well as the quality of the fuels used by the vehicles. Industry is an important source of emissions of suspended particles and heavy metals, while energy production is a key source of emissions of SOx, NOx, suspended particles, and CO. The agriculture sector is the main source category of ammonia (NH_3) emissions. Ammonia emissions mainly result from livestock breeding, fertilizer management, and the use of inorganic nitrogen fertilizers. Agriculture is also a smaller source of emissions of non-methane volatile organic compounds and suspended particles. Suspended particles, especially fine particles, contain microscopic solid or liquid droplets that are so small that they can easily penetrate deep into the lungs and cause serious health problems (Bourennane et al. 2010). Particle size, chemical composition, and physical characteristics define the impact of suspended particles on air quality and human health. Suspended particles also affect climate change (Saeedi et al. 2012).

The processing of ore by processing ore-containing heavy metal emissions generates a local source of emissions in the environment. However, the amount of emissions has decreased significantly in the past two decades as a result of technological development. Also the use of fossil fuels and uncontrolled burning of waste can release heavy metals into the air (Coronas et al. 2013).

Heavy metals in the environment come from natural and anthropogenic sources (Neff et al. 2008). Natural sources of these elements are volcanic eruptions, dust, salts, and fires. Various human activities such as mining, industrial production (smelting, oil refineries, petrochemical plants, and the chemical industry), untreated sewage sludge, coal combustion, and traffic produce far more heavy metals than those that come from natural sources (Fordyce et al. 2005; Goodarzi 2006). Measurement of accumulated metals in lichens is the main method used to assess air pollution by heavy metals. The accumulation process depends on many factors, such as weather conditions (temperature, humidity, wind speed, etc.), the characteristics and health of moss species, and the availability of heavy metal in the environment. There is no doubt about the ability of moss to bioaccumulate heavy metals, as the concentrations measured in their talus are directly related to environmental concentrations. The metal concentration in moss depends primarily on the type of metal in the environment and the physical properties of the metal-containing particles (size and acidity). Different species show different affinities for certain metals, which probably results from the composition and type of binding sites on the cell membrane (Aboal et al. 2010; Balabanova et al. 2010, 2012, 2014; Barandovski et al. 2015).

Anthropogenic and non-anthropogenic activities have increased throughout the world. The consequence is air pollution. Air pollution has created a lot of problems which have affected living and non-living things. To avoid or reduce these problems, constant monitoring of the air should be ensured. The conventional technique is costly because it requires a lot of money and is time-consuming. Biomonitoring has been the alternative method (Markert et al. 2003). Moss, lichens, and plants are biomonitors available to entrap air pollutants. However, different species of mosses have been used for biomonitoring in a number of different ways which may lead to rather different results, and some kind of classification seems necessary at this point. *Epigeic* mosses (growing on the ground) are preferred in the regional surveys in Europe (Fernandez et al. 2007; Harmens et al. 2008, 2010). Trace elements may be either absorbed on the moss from the atmosphere as soluble chemical species in wet deposition or contained in particles from dry deposition. Part of the trace element content of particulates may eventually be released by weathering and reabsorbed by the moss (Onianwa 2001). Whereas uptake efficiencies for particulate-bound trace elements are generally poorly known, ions may be subject to active uptake into cells or attached on the moss surface by physical and chemical forces. Methods are available to distinguish between intracellular and surface-bound fractions of elements. The main problem with the issue of moss biomonitoring is revealed as (a) the transport of soluble compounds from the soil into moss tissue, particularly during periods with excessive soil/water contact (Onianwa 2001). Although mosses do not have a root system, influence from this source cannot be disregarded, in particular in areas with low atmospheric deposition and (b) windblown mineral dust from local soil.

As far as the surface-bound fraction is concerned, little is known about the *binding mechanisms,* but the fact that different metals show rather large differences in their *retention capacities* indicates that both simple *cation exchange* on negative surface charges and *complex formation with ligands* on the moss surface are involved. Furthermore, for the last two decades in developed countries, mosses have been increasingly tested as bio-filters for airborne toxins, with emphasis for the xenobiotics molecules and heavy metals.

5.2 Mosses as Biomonitors

Bryophytes are non-vascular plants (without conductive vessels) that include mosses as a separate group. At the organizational level, bryophytes are found among green algae (of which their offspring are very likely) and among simpler lower vascular plants such as lycopods. Unlike higher plants, the gametophyte (sexual form) is the dominant generation. The sporophyte (asexual form) develops above the gametophyte and remains almost entirely dependent on it. Bryophytes do not have true conductive tissues because they are found in ferns and taller plants (Mägdefrau 1982). Some species of bryophytes are aquatic, and others are able to survive in warm and dry regions. Although its size varies from microscopic to 30 cm, the average bryophyte measures approximately 1.2–5 cm, with color ranging from green to black to almost colorless. Hepatic are the most primitive bryophytes and have a flat shape; sometimes their thickness is only one cell (Mägdefrau 1982).

5.2.1 *Moss Bioindication of Homalothecium Lutescens and* Hypnum cupressiforme

In this study, we will be focused on pleurocarpous moss *Hypnum cupressiforme* Hedw. (cypress-leaved plaitmoss), a common and widespread species of moss belonging to the genus *Hypnum*. It is found in all continents except Antarctica and occurs in a wide variety of habitats and climatic zones. It typically grows on tree trunks, logs, walls, rocks, and other surfaces. It prefers acidic environments and is quite tolerant of pollution. It was formerly used as a filling for pillows and mattresses – the association with sleep is the origin of the genus name *Hypnum*. The genus comprises over 200 species worldwide and 18 in Europe (Mägdefrau 1982). *H. cupressiforme* includes six varieties in Europe (Mägdefrau 1982). *H. cupressiforme* is a small- to medium-sized moss about 2–10 cm long. It is pleurocarpous, having prostrate, creeping stems which form smooth, dense mats. The stems are branched and covered in overlapping leaves giving the impression of a cypress tree. The stem leaves are long and thin measuring 1.0–2.1 mm by 0.3–0.6 mm. They are concave and sickle-shaped, tapering toward the tip. The branch leaves are smaller and narrower than those on the stems. The moss produces short, cylindrical, and slightly curved capsules which contain the spores. The capsules are 1.7–2.4 mm long and have a lid-like operculum measuring 0.6–0.9 mm. They are borne on reddish-brown stalks which are 1–2.5 cm long. The moss is dioicous, having separate male and female plants. *H. cupressiforme* is a highly variable species, and numerous varieties have been described (Mägdefrau 1982).

Homalothecium lutescens is fairly robust, pale, or yellowish-green, with irregularly branched shoots that grow in loose, ascending tufts or as scattered stems. A less common form grows prostrate and closely attached to rocks and boulders, especially on limestone. In both cases, the branches are stout (1–2 mm wide when dry), fairly long (typically more than 1 cm), and straight or nearly so. The leaves are about 2–3 mm long, triangularly spearhead-shaped, widest at the base, and taper evenly to a finely pointed tip. They are distinctive in having very strong pleats lengthways along the leaf, as in *H. sericeum*. The margins are only weakly and finely toothed. Capsules occur less frequently than in *H. sericeum* and are 1.5–2 mm long and slightly curved. Normal forms differ from *H. sericeum* in the long, straight branches and irregular, lax habit. However, prostrate plants on rocks or rocky, coastal banks may only be identifiable if capsules are present (straight, 2–3 mm long in *H. sericeum*; slightly curved and 1.5–2 mm long in *H. lutescens*). Brachythecium glareosum also has strongly pleated leaves, but with a very finely attenuated, hair-like, and often twisted leaf tip. The stems of *Tomentypnum nitens* are covered by a felt of rhizoids, and it occurs in mires and flushes. *Eurhynchium striatum* leaves have longitudinal folds, but are broadly triangular and remain spreading when dry. *Ptychodium plicatum* grows at high altitudes in Scotland and can be distinguished by its numerous miniatures, leaf-like outgrowths on the stem. *Brachythecium albicans* grows with *H. lutescens* in some habitats (e.g., sand dunes) but has more slender, string-like, very pale green shoots, only about 1 mm wide. The leaves of *B. albicans* narrow rather abruptly to a slender tip. *H. lutescens* is characteristic of short, unimproved, calcareous grassland, especially on chalk and limestone. It is also found in other open places, in quarries, and on sand dunes (Mägdefrau 1982).

The focus of this research is on the uses of the two moss species *Hypnum cupressiforme* (Hedw.) and *Homalothecium lutescens* (Hedw.) for monitoring atmospheric heavy metal deposition in lead-zinc mine environ. Sharing the same common name "fern moss" with other monitoring mosses, this species similarly has extensive branching, allowing for a large exposed surface area for ion exchange. These features make *H. cupressiforme* and *H. lutescens* a likely candidate for use as a biomonitor. The primary objective of this study was to evaluate the suitability of two moss species as a bioindicator of heavy metals on a regional landscape scale in the highly polluted area. To accomplish this objective, we tested the availability of *H. cupressiforme* and *H. lutescens* and possibility of applying two types of moss interchangeably to cover denser sampling network. Mosses as pollution bioindicators give only an overview of the areas where we found the presence of high content of toxic metals in atmospheric dust, but not a real measurement of the content in the ambient air. Because of that, the expressiveness of moss species to the metals content was monitored also.

5.2.2 Active Uptake of Metal Ions

Bryophytes accumulate heavy metals by several mechanisms, but the initial and frequently limiting step is reversible adsorption on the cell surface (González and Pokrovsky 2014). Adsorbed metals can be trapped as particulate matter within the surface layer, dissolved in liquids or deposits surrounding cells (intercellular fraction), bound in exchangeable form to exchange or chelating sites on the cell wall and outer surface of the plasma membrane (extracellular fraction), or transported inside the cells and held in soluble or insoluble form (intracellular fraction) (González and Pokrovsky 2014). The extracellular accumulation of heavy metals is mediated by the ion exchange process and the formation of complexes between the metals and the organic functional groups in the cell walls of bryophytes (Shakya et al. 2008). The great binding capacities of mosses for some heavy metals are often attributed to the functional groups of polygalacturonic acid and related polymers in the cell walls (Shakya et al. 2008). Experiments exploring the acid-base properties of the mosses resulted in the detection of several possible functional groups involved in the binding of heavy metals. These include phosphodiester, carboxyl, phosphoryl, and amine groups, as well as polyphenols. Considering the organic composition of the cell walls of mosses, carboxyl and phosphoryl groups could be regarded the dominant metal-binding groups forming the complexes with heavy metals at the surface of moss cells. Other groups, such as sulfhydryl and amine, could be determinants in the presence of small amounts of heavy metals or under extreme pH conditions (González and Pokrovsky 2014). Greater amounts of uronic acids (containing carboxyl groups) in the cell walls compared to cellulose and hemicellulose (having hydroxyl groups) could explain the higher heavy metal binding affinity of the plants cultured in the laboratory than that of the field-grown mosses observed by Wells and Brown (1987). The dominance of carboxyl and phosphoryl groups in the cell walls of different mosses could also explain the similar patterns of heavy metal adsorption in different bryophyte species seen in some studies (González and Pokrovsky 2014; Stankovic et al. 2018). However, it has been observed that adsorbing properties and uptake efficiencies for the same metals may vary significantly between the mosses and liverworts (Shakya et al. 2008). This could be a result of the different cell wall composition of these two bryophyte groups, where uronic acid is a characteristic component of the cell wall of mosses and mannuronic acid in that of the liverworts. Other studies, performed by Rühling and Tyler (1970), have shown that different heavy metals may follow the same order in maximum concentrations reached in the extracellular fractions regardless of the moss species, suggesting that this property depends mainly on the type of the metal. On the other hand, the affinity of extracellular binding sites for different metals may vary significantly among the species. Heavy metals adsorbed on the moss surface can reach the interior of the cell by specific membrane transport proteins or via channels present in the cell membrane (Basile et al. 2012). While the extracellular fraction of heavy metals in mosses is usually easily exchangeable and tends to reflect the current environmental conditions and sporadic peaks in contamination, the intracellular fraction is usually a result of the integration of metals during the longer period of time and thus represents the average situation in the environment (Fernandez et al. 2012). It has been shown, as in other organisms, that the intracellular metal ion uptake by bryophytes displays saturation kinetics (Wells and Brown 1987; Basile et al. 2012). Though it is hypothesized that uptake is a slow metabolically controlled process, the study of Fernandez et al. (2012) revealed that when the bioavailability of heavy metals in the environment is high, intracellular uptake can be rather quick, leading to an accumulation of large amounts of the metals inside the cell in a short period of time. Nevertheless, in this study, the high velocity of heavy metal accumulation inside the cells of the aquatic moss *Fontinalis antipyretica* Hedw. resulted in a quick onset of the release of the same elements into the exterior, suggesting the existence of saturating concentrations inside cells (Fernandez et al. 2012; González and Pokrovsky 2014). Interestingly, in the study of Basile et al. (2012) on different mosses, intracellular concentrations of heavy metals that act as micronutrients, such as Cu and Zn, remained rather constant regardless of their extracellular concentrations, while the accumulation of the elements with no metabolic function, such as Pb and Cd, increased with increasing metal supply in the environment. A similar relationship between the extracellular and intracellular concentrations of Cd was also observed in the moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch. by Fernandez et al. (2012).

One of the potential reasons for the lack of control of non-essential metal input could be the absence of the specific transporters for these metals (Basile et al. 2012). Instead, they could be using channels and transporters of the plasma membrane that normally function in the uptake of essential ions (Wells and Brown 1987;

Basile et al. 2012; González and Pokrovsky 2014), which leads to the increase of their intracellular concentration independent of the previously existent intracellular concentration (González and Pokrovsky 2014). There are numerous sources and factors that can influence the contents of heavy metals in bryophytes. Metals from the atmosphere can reach the surface of terrestrial bryophytes in solution (precipitation) or in the form of dry deposition that can later be solubilized or washed away (Fernandez et al. 2012; Stankovic et al. 2018). Even though terrestrial bryophytes take most of the substances from the atmosphere, soil dust particles contribute significantly to some toxic metal contents (Berg and Steinnes 1997). The bioavailability of the elements and their mobility in soil are significantly correlated to the concentration of the hydrogen ions in soil solution, the organic compounds, and the chemical composition (Salemaa et al. 2004). Windblown particles from the ground containing heavy metals can also influence the amounts of heavy metals in bryophytes (Berg and Steinnes 1997; Salemaa et al. 2004). The retention of these particles on moss surface depends on the particle size and the surface structure (Salemaa et al. 2004; Stankovic et al. 2018). Metal transport from soil to bryophyte depends mainly on the local climatic conditions (Salemaa et al. 2004). Besides these, other sources, such as natural trace element cycling processes and leaching of the heavy metals that were previously accumulated in vascular plants through their root system, may also contribute to the heavy metal content in bryophytes (Salemaa et al. 2004; Stankovic et al. 2018). Water also has a significant role in the heavy metal uptake by bryophytes. For the aquatic bryophytes, it is their living environment and the primary source of all the minerals, including the heavy metals (Salemaa et al. 2004). In the case of terrestrial bryophytes, water can bring or dissolve particles that are already deposited on the bryophyte surface facilitating the uptake of heavy metals by the plant, but it can also wash out the deposited pollutants and lower down the uptake of these elements (Fernandez et al. 2007). The quantity, intensity, and duration of the precipitation determine the amount of accumulated and leached heavy metals from the terrestrial bryophytes. Leaching can significantly influence the uptake of almost all investigated heavy metals. While the uptake efficiencies for metals such as Pb and Ni remain generally stable, leaching process may influence the uptake efficiencies for metals such as Cd, Cu, and Zn or even be a dominant factor in the case of Mn and Cr. The chemical composition of the medium in contact with the bryophyte surface dominantly influences which heavy metal and what amount of it is going to be absorbed and retained by the plant. Different heavy metals differ in their affinities for the binding sites of the cell walls of bryophytes (Rühling and Tyler 1970), indicating that competition effects may significantly alter the uptake kinetics of a specific heavy metal (Wolterbeek 2002; Stankovic et al. 2018).

The concentrations of metals in the environment are usually not high enough to cause the occupation of the majority of the extracellular exchange sites. Conversely, the concentration of protons in strongly acidic environments is high enough and may prevent the binding of different heavy metals by bryophytes or even lead to the leaching of different heavy metals from their cell wall (Salemaa et al. 2004). Wells and Brown (1987) have shown that in the moss *Rhytidiadelphus squarrosus* (Hedw.)

Warnst., lowering of pH not only reduces the extracellular binding of Cd, but it also affects its intracellular uptake. While the first could be due to the protonation and occupation of the available extracellular anionic binding sites, the second could be a result of the proton-induced conformational changes of transporting proteins in the bryophyte membranes. Thus, the type (soil, air, or water) and chemical composition of the media and its acidity are probably the most important factors determining which metal and what amount of it is going to be accumulated by different bryophyte species (Stankovic et al. 2018).

Sources of heavy metals can be of anthropogenic origin and contribute to their spread to rural areas and agricultural land (metal processing industry, electroplating industry, ore smelters, as well as burning of fossil fuels, landfills of urban and industrial waste, sewage sludge). Toxic metals can contaminate agricultural land in the process of fertilization with sewage sludge (Cd, Ni, Cu, Pb, Zn), as well as the use of phosphate and organic fertilizers (Cd, Cr, Mo, Pb, U, V, Zn, etc.) and pesticides (Cu, As, Pb) which is why the application of these agents must be under strict control, because heavy metals are found in them as impurities (Bourennane et al. 2010). Soil treated with such agents in agricultural regions is subjected to monitoring for potentially hazardous metal inputs.

5.2.3 Toxic Metal Accumulation in Bryophyte

Heavy metals are transition metals with a density over 5 g/L and show marked environmental toxicity. In the environment, they are found in air, water, food, and soil. Certain heavy metals, such as iron, cobalt, copper, zinc, and manganese, are necessary for numerous functions and normal growth and development of living organisms and are labeled as essential (Martin and Coughtrey 1982). However, the accumulation of non-essential minerals (such as heavy metals: cadmium, lead, arsenic, mercury) in living organisms can result in reduced normal growth and development, acute poisoning, physiological changes, and even death. Their origin can be natural (geochemical) and anthropogenic (Ajmone-Marsan et al. 2008). Rock decomposition occurs through the process of pedogenesis, and hydration, hydrolysis, dissolution, oxidation, or reduction releases heavy metals from the parent substrate. A part of heavy metals in the soil is formed as a result of geochemical processes, but also volcanic eruptions and continental dust dispersal. In the form of dust particles, they reach the atmosphere and settle in water and soil. Heavy metals precipitate as sparingly soluble carbonates, sulfates, or sulfides, and their toxicity is increased by the process of chelation and formation sulfides with biologically active substances (especially with enzymes) in a process termed biomethylation (Fordyce et al. 2005).

Heavy metals have become one of the main abiotic factors with a negative effect on living organisms. By consuming, plants play a significant role in the circulation of toxic metals because they enter the food chain and thus negatively affect human health. The accumulation and availability of toxic metals are especially influenced by the type of soil, pH value, as well as the content of organic matter in the soil (Stankovic et al. 2018). Representing parts of some enzyme complexes and proteins, essential heavy metals are necessary for normal plant growth and development. However, the mentioned accumulation of heavy metals can be a consequence of binding to SH groups of proteins and result in inhibition of enzyme activity, disruption of their structure, deficiency of essential metals, and stimulation of production of free radical species, causing oxidative stress (De Miguel et al. 1999). Accumulation of heavy metals causes inhibition of growth of aboveground and underground parts, reduction of seed germination, reduction of photosynthetic pigments, formation of chlorosis and leaf necrosis, and loss of turgor, which is related to accelerated aging and death of the plant itself. Toxic metals lead to changes in the structure of plant tissue and biochemical and molecular processes (Järup 2003).

5.2.4 The Effects of Heavy Metals on Bryophytes

Though growth and development are commonly used parameters for the assessment of the heavy metal toxicity in plants, negative effects of heavy metal pollution could be detected before the alteration of these two parameters become obvious (Wolterbeek 2002). These effects include ultrastructural changes as well as the changes in the plant physiological processes and characteristics (Stankovic et al. 2018). Ultrastructural changes seen in bryophytes under heavy metal stress are evident (Stankovic et al. 2018). Along with the ultrastructural changes, heavy metals may also disrupt various metabolic processes. These negative effects could be explained by the high affinity of heavy metals for sulfhydryl groups in various proteins (Boquete et al. 2014; Stankovic et al. 2018). The chlorophyll content can be also used as a tracking signal for assessment of the physiological state and biochemical functionality of plant organism (Nagajyoti et al. 2010). However, there have not been many studies investigating the relationship between the presence of different heavy metals in bryophytes and the chlorophyll concentration (Stankovic et al. 2018).

5.2.5 Intracellular and Surface-Bound Fractions of Elements

Just like other terrestrial plants, mosses have adapted to life on land: the waxy cuticle is a protection against dehydration, gas exchange takes place through the coup, and their zygote remains protected within the female sexual organ (archegonia) developing into a multicellular embryo. Mosses with terrestrial plants share some other significant features: they are parenchymal structures, and their life cycle consists of heteromorphic and heterophase generation change (Leblanc and Rao

1974). However, mosses also have some specific characteristics that we do not find in other terrestrial plants, which really makes them a special plant group. They are non-vascular plants, meaning they do not have a conduction system in the form of phloem and xylem, for the supply of water and nutrients. Therefore, all mosses can absorb water all over the body (González and Pokrovsky 2014). Mosses also lack an internal support system because their cell wall does not contain the solid substance lignin, commonly present in stem cell conduction cells at a higher stage of development. The vegetative body of moss is not differentiated into three basic parts, root, stem, and leaf, which are found in other stems, but there are analogous formations. Instead of roots, most mosses have thin, hair-like tubes, which are also found in algae and are called rhizoids (Mägdefrau 1982). They attach the body of the moss to the substrate and supply nutrients from the soil. The part that corresponds to the stem is called the stems, while instead of leaves in mosses we are talking about leaves (philoids). Depending on the appearance of the tree, mosses can be divided into two groups: (1) sedimentary mosses, which have a sedimentary body resembling algae (it is not separated into stems and leaves, but is patchy and laid down and attached to the substrate by means of rhizoids), and (2) folio mosses in which the vegetative body is divided into stems and leaves and instead of roots they also have rhizoids. Unlike other stem plants, mosses have a unique life cycle in which the vegetative green body of the plant is a gametophyte or sexual generation and as such is dominant over the sporophyte or asexual generation. The small sporophyte, which has no special assimilation organs, parasitizes on the gametophyte and is physiologically completely dependent on it. In more sedentary trees, the situation changes significantly, and there are a trend of reducing gametophytes and the simultaneous development of sporophytes in the dominant generation (Mägdefrau 1982).

The toxic effects of heavy metals in bryophyte cells are predominately caused by the intracellular fraction, while the metals outside the cells do not have immediate effects on cellular metabolism (Fernandez et al. 2007; Shakya et al. 2008; Basile et al. 2012). Thus, the strategies used by bryophytes in response to heavy metal stress may include both the avoidance and the tolerance of this type of abiotic stress. Modification of any of the characteristics influencing its retention and cation exchange capacities or the activity of metal transporters in plasma membrane could lead to exclusion of heavy metals (Boquete et al. 2014). For example, differences in the cell wall chemical composition between the mosses and liverworts or in different species in a group could explain the differences in the uptake of different metals and thus the differences in their sensitivity to these pollutants observed in Stankovic et al. (2018). They have shown that the degree of tolerance to cadmium may be influenced by the cell wall binding of different non-toxic cations naturally occurring in the cells or the environment, which then can create unfavorable conditions for the binding of heavy metals around the plasma membrane and prevent their entrance into the cytoplasm.

5.3 Dry vs. Wet Deposition

For several decades, it has been clear that humanity, in addition to extraordinary successes in many areas of science and technology, is facing environmental threats. Of particular concern are anthropogenic emissions of pollutants into the atmosphere in which complex chemical reactions and dynamic processes take place. Substances emitted into the atmosphere are subject to certain transformation processes over time, with the formation of new compounds that are deposited on the earth's surface together with the originally emitted substances. This deposition takes place without the participation of water molecules (dry deposition) or with the participation of water molecules (wet deposition). This leads, in addition to the phenomenon of cleaning the atmosphere, to certain problems in the environment such as acidification of soil and water systems and damage to biological materials, endangering human health.

The theoretical processes of dry and wet deposition are very complex. However, atmospheric pathways and the characteristics of standard and non-standard pollutants are much better described for aerosols than for gaseous substances, for both wet and dry deposition. Wet deposition refers to acid rain, fog, and snow. All these acidic precipitations outside and through the ground affect plants and animals. The strength of this influence depends on a number of factors, the degree of acidity of the water, chemical and receiving capacity of the soil, and the type of trees, fish, and other organisms in water (Ajmone-Marsan et al. 2008). Dry deposition refers to acid gases and particles. About half of the acidity in the atmosphere falls to earth in the form of dry deposition. The wind causes acid particles and gases on buildings, cars, houses, and trees. Dry deposited gases and particles can also be washed away by rain, making the water even more acidic. Dry deposition refers to gases and particles, is independent of atmospheric precipitation, and is regulated by the concentration of pollutants in the air and transport through the boundary layer, the chemical and the physical nature of the depositional species, and the efficiency of the surface that accepts or absorbs the deposited material. It is part of the total exchange of matter between the atmosphere and the earth's surface, all the more so because the deposited gases are sometimes reversibly absorbed so that, under certain conditions, they can be re-emitted into the atmosphere (White 2013; Keller et al. 2015). Direct measurement of dry deposition is complex and very rarely performed in the world and is estimated by mathematical modelling (Bourennane et al. 2010).

Dry and wet deposition of pollutants in the gaseous state and particles take place in different ways. The basic characteristics of dry deposition are that the vertical concentration gradient pushes the molecules toward the soil, thus enabling their adsorption in the first layers of the soil, or they are retained in the layers of air that move laminarly in their direct contact with the soil or chemically react with molecules that are on the ground (Gjengedal and Steinnes 1990).

For wet gas deposition, it is characteristic that pollutants reach the ground by washing with snow or rain. These compounds primarily reach the clouds where chemical reactions take place, followed by leaching in the form of droplets and snowflakes. Suspended particles with a diameter of less than 10 μm have a different deposition path, since they have a negligible mass and are therefore more susceptible to the influence of gravity than gases. A significant part of the suspended particles is deposited by sedimentation. Dust particles are also deposited together with a larger number of particles of lower density, such as gaseous particles (Tye et al. 2006). A different mechanism of dry deposition takes place when a particle enters an element of air volume and, carried by the wind, reaches the ground. Instead, wet deposition mainly occurs when particles act as nuclei of the condensation process for water molecules that turn into rain or snow. Gases present in the atmosphere can be removed by atmospherics, precipitation by rain, snow, and ice.

5.4 Fate of Aerosols

The fate and behavior of substances in the environment depend on the physicochemical characteristics of the substance itself and the environment in which the observed substance is located. The atmosphere contains a significant amount of aerosols, from droplets of fog or clouds to dust particles from the ground and smoke from combustion, which greatly affect the fate and behavior of certain substances in the environment. Atmospheric particles can have different physical and chemical characteristics such as size, concentration, chemical composition, hygroscopy, density, morphology, and electrical and optical characteristics; although significant variability in particle size has been observed, their diameter is, in most cases, a few μm. Fine particles are emitted by combustion and form ultrafine and accumulative particles. Ultrafine particles with a diameter, Dp, below 0.1 μm are formed in a homogeneous atmosphere by nucleation or coagulation of smaller particles, while accumulative particles ($0.1 < Dp < 2.0$ μm) are formed by condensation on smaller particles. Large particles, larger than 2 μm, are formed mechanically, by separation from larger solid and liquid surfaces. Aerosol concentration is most often expressed in μg/m^3. In rural areas, the concentration usually does not exceed 5 μg/m^3, while in polluted areas in urban areas, the aerosol concentration is about 100 μg/m^3 (Mackay 2001). Atmospheric aerosol originates both from natural sources, such as "sea dust," soil particles emitted into the atmosphere by wind, transboundary transport of dust from the Sahara, and volcanic and biogenic emissions, and from anthropogenic activities, such as burning fossil fuels in industry, traffic, and households. Depending on the mode of emission from these sources, the aerosol is classified into primary and secondary – the primary aerosol is emitted directly into the atmosphere, while the secondary aerosol is formed by condensation, followed by coagulation, as a result of oxidation of hydrocarbons in the atmosphere to less volatile compounds. In air with a dominant emission of the primary aerosol, a significant amount of ultrafine particles is present, while in the air that makes up the secondary aerosol, accumulative particles coated with a liquid organic and inorganic film will dominate.

Soot emitted by diesel engines and the combustion of fossil fuels for household heating is a source of primary organic elemental carbon in the aerosol. Elemental carbon (EC) has a chemical structure similar to impure graphite and is emitted during the combustion process as a primary particle. Oxidation processes in the gaseous phase convert reactive hydrocarbons (terpenes and aromatic compounds) into semi-volatile organic compounds, which then bind to the aerosol's particles, primarily in urban areas where the mentioned emission sources dominate.

5.4.1 Atmospheric Depositions of Heavy Metals

The presence of heavy metals in the soil is a consequence of natural and anthropogenic processes. There are natural pedogenetic processes by which the soil inherits heavy metals from the parent substrate, and anthropogenic processes include urbanization, industrialization, trade, and agricultural production. In remote areas with low anthropogenic impact on atmospheric deposition, heavy metals in soils are mostly derived from the parent substrate, while in urban and agricultural areas, the concentrations of heavy metals in soils are higher than concentrations in parent substrates due to continuous input into the ecosystem. The geogenic origin of the most ecologically interesting heavy metals, Cu, Zn, Cd, and Pb, is most often associated with sulfur minerals, which oxidize relatively quickly in the environment, and the metal cation separates from sulfur at an early stage of mineral depletion (He et al. 2005). In the later stages of pedogenesis, Cu, Zn, and Cd are more often in the composition of Mn oxide, and Pb is in the composition of Fe oxide and hydroxide. The average proportion of igneous and sedimentary rocks in the earth's crust is 95:5, and sedimentary rocks are more common in the surface layers. Soils formed on sandstones and acidic rocks (e.g. granite) usually contain less essential elements and heavy metals Cu, Zn, and Co (He et al. 2005) than alkaline rock and sedimentary shale soils (may contain more than 200 mg/kg Cd). Natural geological processes on different parent rocks can result in multiple concentrations of heavy metals compared to the soil average, affecting flora and fauna. Natural sources of heavy metals in the soil are, in addition to parent rocks, volcanic eruptions, marine aerosols, and forest fires (Keller et al. 2015).

Agricultural production contributes to the accumulation of heavy metals in the surface layers of agricultural soils by applying various agrotechnical measures:

1. Fertilization with mineral fertilizers (natural minerals, individual and complex fertilizers)
2. Fertilization with organic fertilizers (manure, compost, organic residues)
3. Soil conditioning (calcification, acidification, texture improvers)
4. Application of pesticides
5. Irrigation and fertigation

The use of various substances containing metals has significantly increased in order to achieve stable yields, and the application of microelements such as Cu, Zn, Fe, Mn, and B has become a common agrotechnical intervention. These elements are regularly added to the usual formulations of complex fertilizers when fertilizing crops on sandy, carbonate, and peat soils with a lack of trace elements (He et al. 2005). A significant part of pesticides, fungicides, and herbicides also contain Cu, Zn, Fe, Mn, and even As, and some heavy metals such as Cd and Pb are introduced into the soil as impurities present in fertilizers. Phosphate fertilizers have the greatest importance among mineral fertilizers in terms of heavy metals as impurities, i.e., crude phosphates as single fertilizers or as raw material for the production of single and complex fertilizers. We pay the greatest attention to the concentration of Cd in phosphate minerals, although the share of other heavy metals is very significant. Significant concentrations of heavy metals are found not only in natural phosphates but also in various mineral fertilizers, conditioners, organic fertilizers, and residues in agricultural production. Organic fertilizers such as manure and compost contain higher concentrations of heavy metals than most agricultural soils. The consequence of continuous use of organic fertilizers is an increase in the total concentration of heavy metals in the soil. There is a significant positive effect of fertilization on the availability of essential heavy metals, especially on light sandy and carbonate soils, but the negative consequence is an increase in the concentration of toxic heavy metals such as Cd and Pb. Therefore, in most countries, the maximum concentration of certain heavy metals in organic fertilizers is prescribed.

5.5 Chemometric Approaches for Identification of Metal Depositions

As bioindicators, moss was first used in the Scandinavian countries in the late 1960s, as a complementary method to classical instrumental methods for testing and controlling environmental pollution (Rühling and Tyler 1968). Later, mosses and lichens were used as indicators of radioactive contamination of the environment to examine the global deposition of radionuclides as a result of nuclear tests in the atmosphere, to assess the consequences of the Chernobyl nuclear accident in 1986, and to assess the state of the environment around nuclear plants and reactors, nuclear fuel and material processing plants, etc. Today, the moss biomonitoring method is part of the environmental control program in most European countries because it provides data on anthropogenic pollution in urban areas, as a consequence of traffic and fossil fuel combustion, and allows the identification of sources of heavy metal pollution (Harmens et al. 2008, 2010). Moss is a suitable indicator for the detection of long-term pollution with uranium and other heavy metals because it accumulates uranium and other elements and retains them for many years after pollution stopped (Harmens et al. 2008, 2010; Balabanova et al. 2010, 2016; Bačeva et al. 2012; Barandovski et al. 2015). However, mosses are often difficult to find in urban areas, especially in quantities sufficient for analysis. Therefore, the technique of so-called active biomonitoring or bugs (bags technique) with moss transplants has significant advantages, such as precisely defined exposure time, known concentration of elements in mosses, and flexibility of site selection (Barandovski et al. 2015). The main disadvantages of the method are ignorance of the efficiency of accumulation for different types of pollutants, as well as the choice of exposure time.

Biomonitors are organisms, parts of organisms, or a community of organisms that are exposed to different types of pollution and provide information on the quality and condition of the environment. They can also be used to detect the negative impacts that various anthropogenic activities (industrial activity, transport, etc.) have on the environment. The information obtained can make a significant contribution to sustainable development and the development of strategies to prevent or reduce adverse environmental impacts.

Each organism in the ecosystem can give us information about the state of its environment. Many plant species show sensitivity to various pollutants. The degree of their sensitivity to pollutants varies depending on the species. According to numerous previous researches, mosses have proven to be very good biomonitors of air pollution. Moss (Bryophyta) is a primitive in evolutionary sense, but a widespread group of terrestrial plants. In most cases, mosses are perennial plants ranging in size from a few millimeters to 60 cm. Unlike “higher” plants, mosses do not have a developed true root system, stem, and leaf, but only analogous parts that resemble the mentioned organs. Due to the non-existence of the cuticle on the surface of the leaves, mosses absorb water and nutrients from the air and thus pollutants. Thanks to these morphological and physiological properties, mosses are good biomonitors of pollutants, primarily heavy metals, present in the surrounding air.

Heavy metals can reach the environment from anthropogenic sources, industry and traffic, and to a lesser extent from natural sources: volcanic eruptions or forest fires. Heavy metals, as pollutants in the living and working environment, are a serious health and environmental problem because they are toxic and non-biodegradable and have a very long stay in the environment. Active biomonitoring is another research methodology in determination of the millimeter atmospheric depositions. Active biomonitoring is the removal of mosses from their natural habitat and exposure to locations from which they are absent – urban areas, industrial zones, and other polluted areas – in order to monitor air quality. There are several proposed chemometric interpretations of data obtained from this kind of investigations:

Moss Accumulation Index to assess the accumulation of each toxin by moss species, relative accumulation factors (RAF) will be calculated using Eq. (5.1):

$$\mathrm{RAF} = \left(C_{\mathrm{exposed}} - C_{\mathrm{initial}}\right) / C_{\mathrm{initial}} \tag{5.1}$$

where C_{exposed} is the content of each element after exposure and C_{initial} is the content before exposure. Contamination factors (CF), used to assess the degree of anthropogenic influence, will be calculated using Eq. (5.2):

$$\mathrm{CF} = C_{\mathrm{moss}} / C_{\mathrm{background}} \tag{5.2}$$

Artificial Neural Networks (ANN) ANN are defined as structures comprising densely interconnected adaptive simple processing elements, called artificial neurons (or nodes), which can perform massively parallel computations for data processing and knowledge representation.

Classification and Regression Trees (CARTs) CARTs is a powerful and flexible classification tool. It handles both ordered and categorical predictor variables. The final classification rule has a simple form, which is easy to interpret and to use for future classification. CARTs takes into account the fact that different relationships may hold between variables in different parts of the data. It does automatic stepwise variable selection and calculates the importance rank of the variables. CARTs calculates misclassification error estimated by both re-substitution and cross-validation.

Linear Discriminant Analysis (LDA) LDA is probably the most frequently used supervised pattern recognition method and the most studied one. LDA is based on the determination of linear discriminant functions, which maximize the ratio of between-class variance and minimize the ratio of within-class variance. In LDA, classes are supposed to follow a multivariate normal distribution and be linearly separated. LDA can be considered, as PCA, a feature-reduction method in the sense that both, LDA and PCA, determine a smaller dimension hyperplane on which the points will be projected from the higher dimension.

Cluster Analysis (CA) clustering is the task of assigning a set of objects into groups (called clusters) so that the objects in the same cluster are more similar (in some sense or another) to each other than to those in other clusters. In this way, we categorize the samples in order to succeed with discrimination.

Principal Component Analysis (PCA) PCA is a technique that, by the reduction of the data dimensionality, allows their visualization while retaining as much as possible the information present in the original data. So, PCA transforms the original measured variables into new uncorrelated variables called principal components (PCs). Each PC is a linear combination of the original measured variables. This technique affords a group of orthogonal axes that represent the directions of greatest variance in the data. The first PC (PC1) accounts for the maximum of the total variance, the second (PC2) is uncorrelated with the first and accounts for the maximum of the residual variance, and so on, until the total variance is accounted for.

Analysis of Variance (ANOVA) ANOVA is a collection of statistical models, and their associated procedures, in which the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether or not the means of several groups are all equal and therefore generalizes t-test to more than two groups. Performing multiple two-sample t-tests would result in an increased chance of committing a type I error. For this reason, ANOVA is useful in comparing two, three, or more means and has been used to compare elemental profiles of foods of different origin.

5.6 Bioindication Screening Model

Certain surveys, especially in larger areas, require quick and easy screening of a given area. Analytical techniques are absolutely dominant when it comes to quantification of chemical elements. But usually before setting up the monitoring experiment, it is necessary to scan a larger area in order to obtain semi-quantitative information about the condition of the selected area. Rapid screening will aim to provide information on where the most effective sub-areas are, where careful sampling network will need to be carefully generated later. Therefore, the aim of this research is to propose a fast hemometric model for scanning contaminated areas with heavy metals. This model is particularly applicable to large areas or areas where certain incidents have occurred, for which the effect of pollution has yet to be determined. To better understand the factors leading to metal binding in complex moss matrix and trace metal fate, electron microscopic characterization of samples will be conducted. Scanning electron microscopy can reveal the presence of trace metal-specific incorporation in organic matrix in close association with biological structures with consistent morphologies. For the present investigation, scanning electron microscope model Hitachi (model SU8320) will be used in order to characterize the morphology, structure, and metal binding mechanisms. Furthermore, another approach will be introduced for semi-quantitative screening of moss surface. Preliminary investigation has been implemented for improving this technique for monitoring of surface dust physical transport.

5.6.1 Moss Sample Preparation

Three moss samples of *Hypnum cupressiforme* (Hedw.) and *Homalothecium lutescens* (Hedw.) were collected in the period of September–October 2012, at the critical mining areas in the Eastern part of the Republic of North Macedonia (copper mine and Pb-Zn mine). These samples were used in similar investigation as given by Balabanova et al. (2017). Three sampling points were selected: two critically affected and one control site. Depending on the conditions and the accessibility of the locations, the species that is available and typical for the region was collected. The moss sampling protocol was performed according to the set standard rules for collection of such samples. The whole procedure was done in this order: one sampling spot is formed by collecting five sub-spots in the area of 50 × 50 m^2. Every spot of sampling network must be in a distance of minimum 300 m from main roads, 100 m from local roads, and 200 m from villages. Moss samples were collected using polyethylene gloves, to prevent contamination of any further samples. The collected material was stored in paper bags. After it was cleaned from other plant species, soil individual plant samples are separated and air-dried for several days. Dry samples were again placed in paper bags until analyses were performed.

5.6.2 SEM Analysis

The scanning electron microscopy (SEM) is used for observation of specimen surfaces. When the specimen is irradiated with a fine electron beam (called an electron probe), secondary electrons are emitted from the specimen surface. Topography of the surface can be observed by two-dimensional scanning of the electron probe over the surface and acquisition of an image from the detected secondary electrons.

For this pilot research, double-sided adhesive carbon tape is applied to the sample stub, to which a small amount of the sample is attached. The sample is made of gold; the coating is up to 20 nm thick. The sample thus prepared is placed in the chamber of a scanning electron microscope, and the necessary analyses are performed by achieving a high vacuum. The analyses were performed using SE (secondary detector) at 30 kV voltage. SEM analyses were conducted in the National Institute for R&D of Isotopic and Molecular Technologies, Integrated Electron Microscopy Laboratory, in Cluj, Romania.

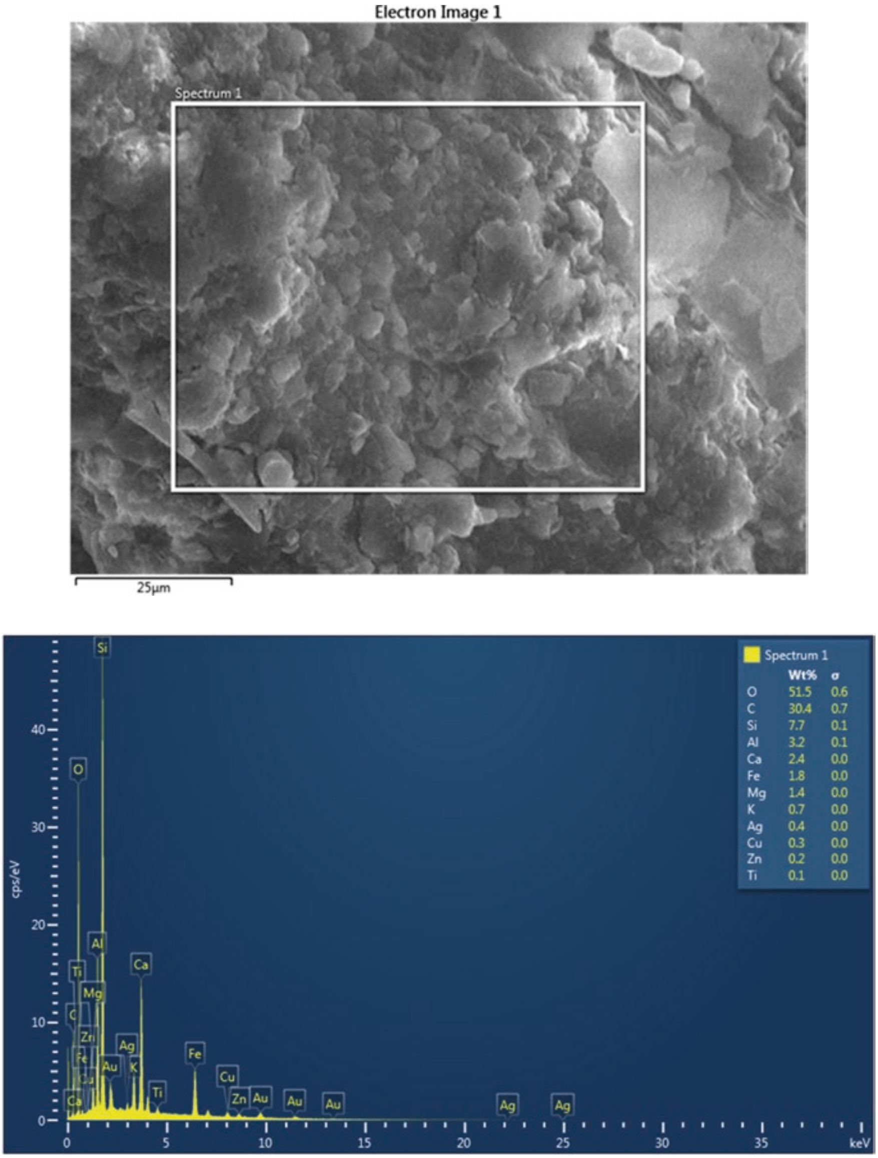
5.7 Data Summary and Perspective

5.7.1 Moss Sample SEM Analysis

The energy-dispersive X-ray spectrometer (EDS) is used to analyze characteristic X-ray spectra by measuring the energies of the X-rays. When the X-rays emitted from the specimen enter the semiconductor detector, electron-hole pairs are generated whose quantities correspond to the X-ray energy. Measuring these quantities (electric current) enables to obtain the values of X-ray energy. The detector is cooled by liquid nitrogen, in order to reduce the electric noise. The advantage of the EDS is that the X-rays from a wide range of elements from B to U are analyzed simultaneously. The following figures present the X-ray spectrum obtained from EDS for the analyzed moss samples. Moss surface screening can give us very useful information of the dust composition and moss tissue accumulation of potentially toxic elements. Given in Figs. 5.1, 5.2, and 5.3 are the qualitative and quantitative chemical analyses of the surface of the *Hypnum cupressiforme* moss samples (25, 50, and 100 μm). Figures 5.1b, 5.2, and 5.3b represent the following: the X line is the spectral analysis-qualitative analysis element detection, and the Y line is the cps (counts per seconds), the quantitative analysis determination of the concentration. Figure 5.4a, b represents the comparison of two spectra of the surface, spectrum 3 (yellow) and spectrum 4 (red) lines, and the differences of the elementary composition into the samples. Spectrum of gold is visible in every analysis, but it does not count, because the sample is coated with gold so the quantitative analysis of gold is not showed into the figure (settings conducted before each analysis). Figures 5.1, 5.2, 5.3, and 5.4 are the screening analysis of the *Hypnum* sample representing as a control sample (in non-polluted area). The SEM analyses represented in Figs. 5.5, 5.6, 5.7, 5.8, and 5.9 are the screening of the *Homalothecium lutescens* moss sample. Figure 5.6a, b represents the SEM analysis of *Homalothecium lutescens* sample from the polluted site where two analyses are compared: one is point analysis as spectrum 9 (yellow) and one is surface analysis as spectrum 10 (red). Figures 5.7, 5.8, and 5.9 represent the qualitative and quantitative chemical analysis of the surface of the *Homalothecium lutescens* moss samples (100, 250, and 500 μm). Figures 5.10, 5.11, and 5.12. Comparative analysis of two surface screening, spectrum 15 (yellow) and spectrum 16 (red), is presented in Fig. 5.11a, b, for *Hypnum cupressiforme* sample from a polluted site. The three samples were scanned and surface absorption was monitored. Advanced scans help to better identify the particles and how they are retained in the moss tissue. For each SEM analysis, a chemical characterization was performed for the given scan. But what is very important for this research is the analysis of the moss surface compared to the substance/substance absorbed on the moss surface. Such comparisons are made for the control sample (Fig. 5.4a, b). Spectrum 4 detects the tissue surface of the moss sample, while spectrum 3 is the absorbed dust. The chemical characterization is dominant for the biogenic elements, while the deposited matter is dominated by iron, aluminum, and silicon. The other two specimens are from a potentially contaminated area, but are different species. Therefore, the analysis was aimed at comparing the two types as well as the possibility of expression of anthropogenic elements in the deposited dust. The dual surface analysis of *Homalothecium* moss is presented in Fig. 5.6a, b. The scan shows two spectra, one from the surface of the tissue (spectrum 10) and a part where the presence of a deposit has been determined (spectrum 9). Biogenic elements dominate the chemical characterization (C, O, and Si), while dust shows a charge for several anthropogenic elements. Analysis of *Hypnum cupressiforme* showed more significant expression in the identification of the elements contained in the deposited dust. Two identifications were made, presented in Fig. 5.11a, b, where only a difference in biogenic elements is observed between the moss tissue (spectrum 16) and the deposited matter that is not enriched with anthropogenic elements (spectrum 15). The analysis of both spectra 13 and 14 (Fig. 5.12a, b) identified a deposited particle with a significant difference in the content of Cu, Fe, and Zn, which is characteristic of the mine environment for the exploitation of copper minerals.

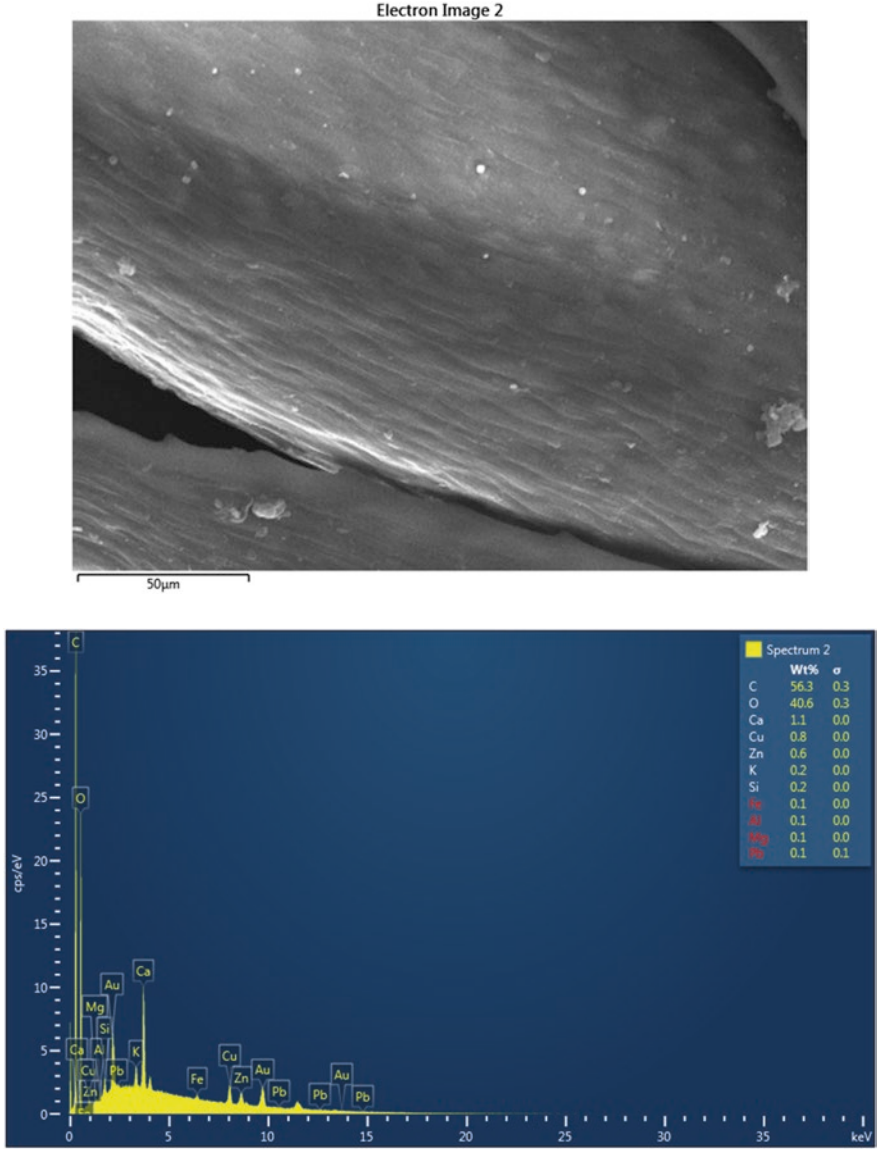
5.7.2 X-Ray Mapping of Moss Samples and Dust Particles

X-ray mapping is used to obtain the distributions of specific elements. In this analysis, the electron probe is scanned over a specified area, and characteristic X-rays with specific energies are acquired. It should be noted that if the P-B ratio is extremely low (the peak intensity is very small compared to the background), X-ray maps show the distribution of continuous X-rays (not the distributions of elements of interest). Energies of characteristic X-ray elements not of interest are very close to those of the elements of interest; X-ray maps might show the distributions of the



	Wt%	σ
O	51.5	0.6
C	30.4	0.7
Si	7.7	0.1
Al	3.2	0.1
Ca	2.4	0.0
Fe	1.8	0.0
Mg	1.4	0.0
K	0.7	0.0
Ag	0.4	0.0
Cu	0.3	0.0
Zn	0.2	0.0
Ti	0.1	0.0

Fig. 5.1 (**a**) Surface analysis of *Hypnum cupressiforme* – control sample (25 µm). (**b**) Chemical composition of *Hypnum cupressiforme* for the scan given in Fig. 5.1a



	Wt%	σ
C	56.3	0.3
O	40.6	0.3
Ca	1.1	0.0
Cu	0.8	0.0
Zn	0.6	0.0
K	0.2	0.0
Si	0.2	0.0
Fe	0.1	0.0
Al	0.1	0.0
Mg	0.1	0.0
Pb	0.1	0.1

Fig. 5.2 (**a**) Surface analysis of *Hypnum cupressiforme* – control sample (50 µm). (**b**) Chemical composition of *Hypnum cupressiforme* for the scan given in Fig. 5.2a

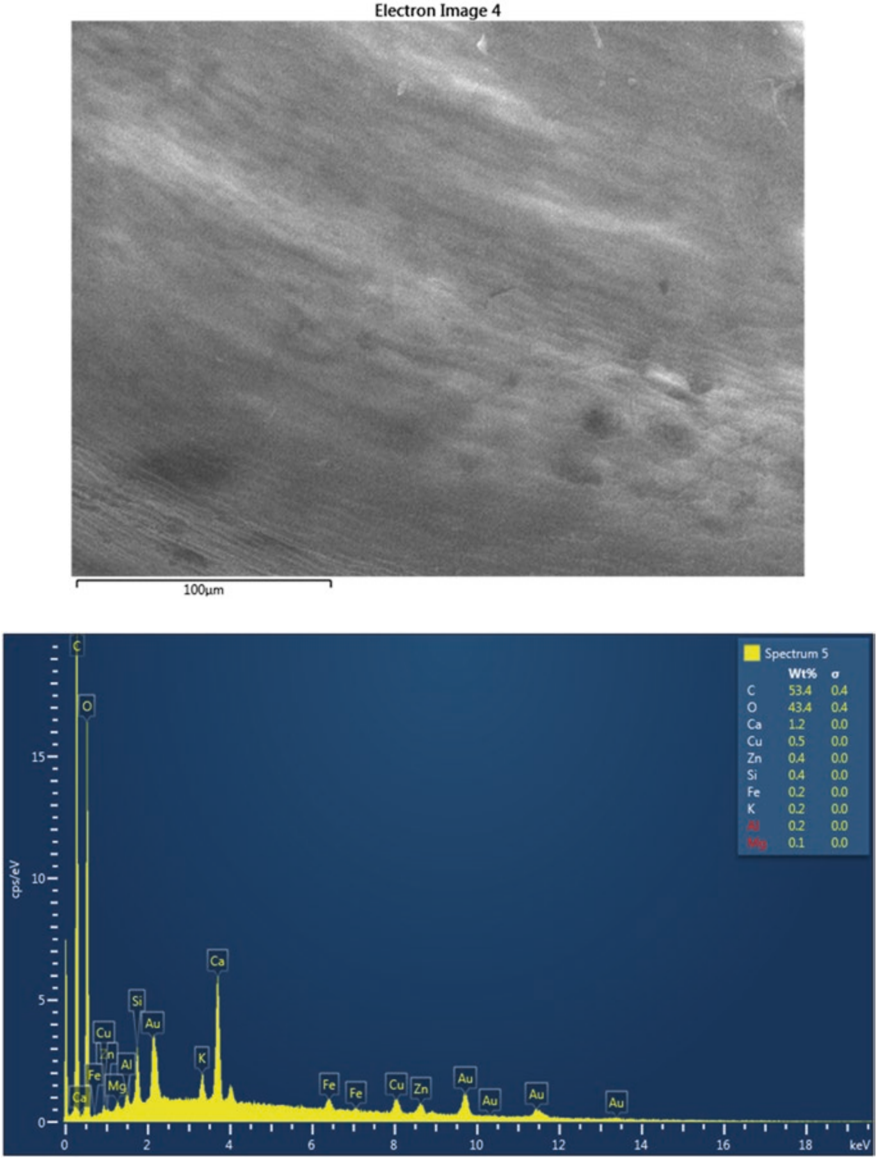
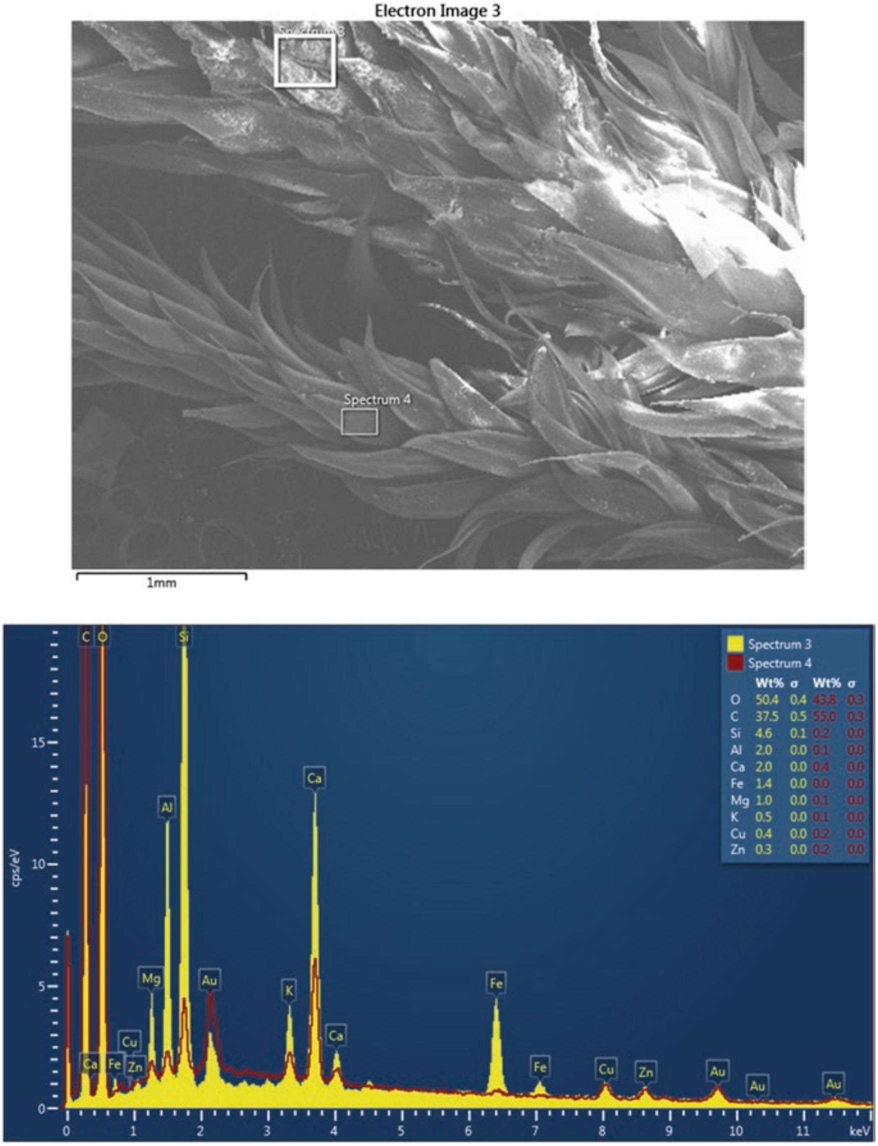
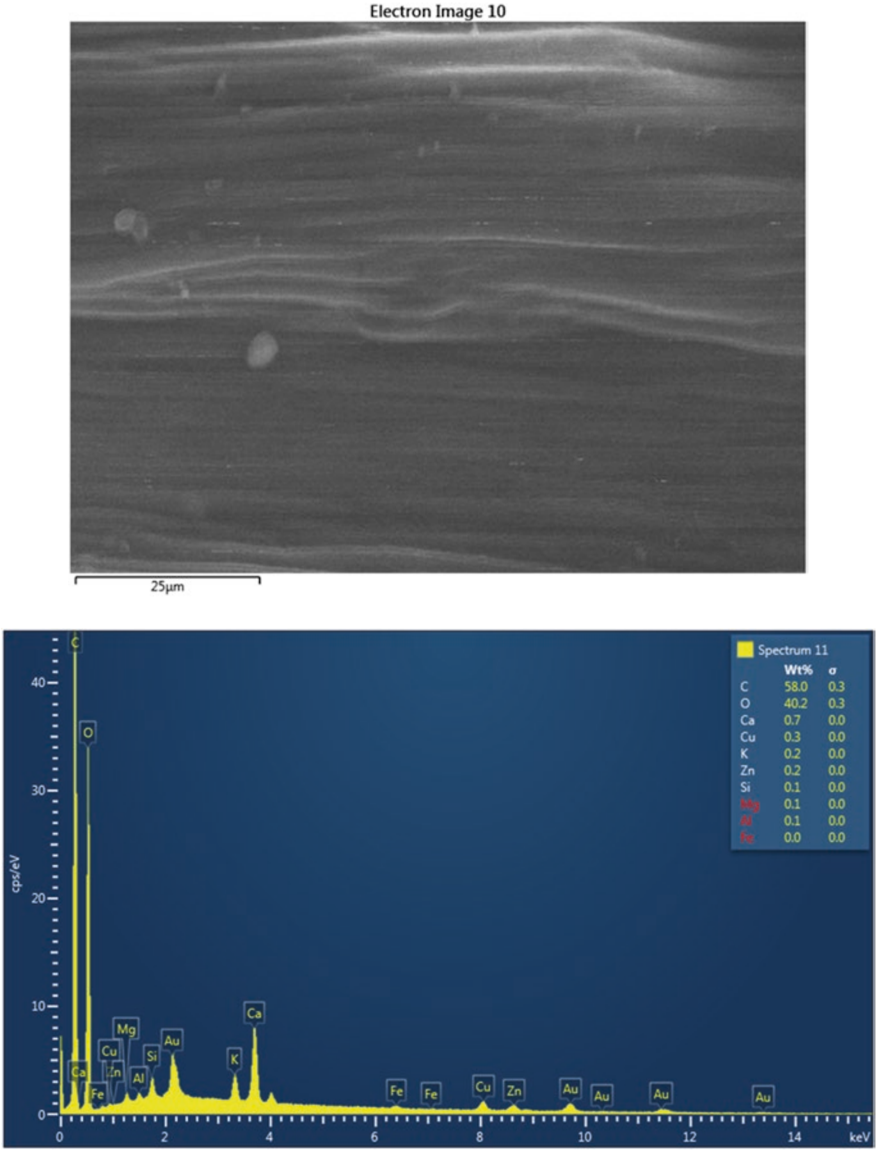


Fig. 5.3 (**a**) Surface analysis of *Hypnum cupressiforme* – control sample (100 µm). (**b**) Chemical composition of *Hypnum cupressiforme* for the scan given in Fig. 5.3a



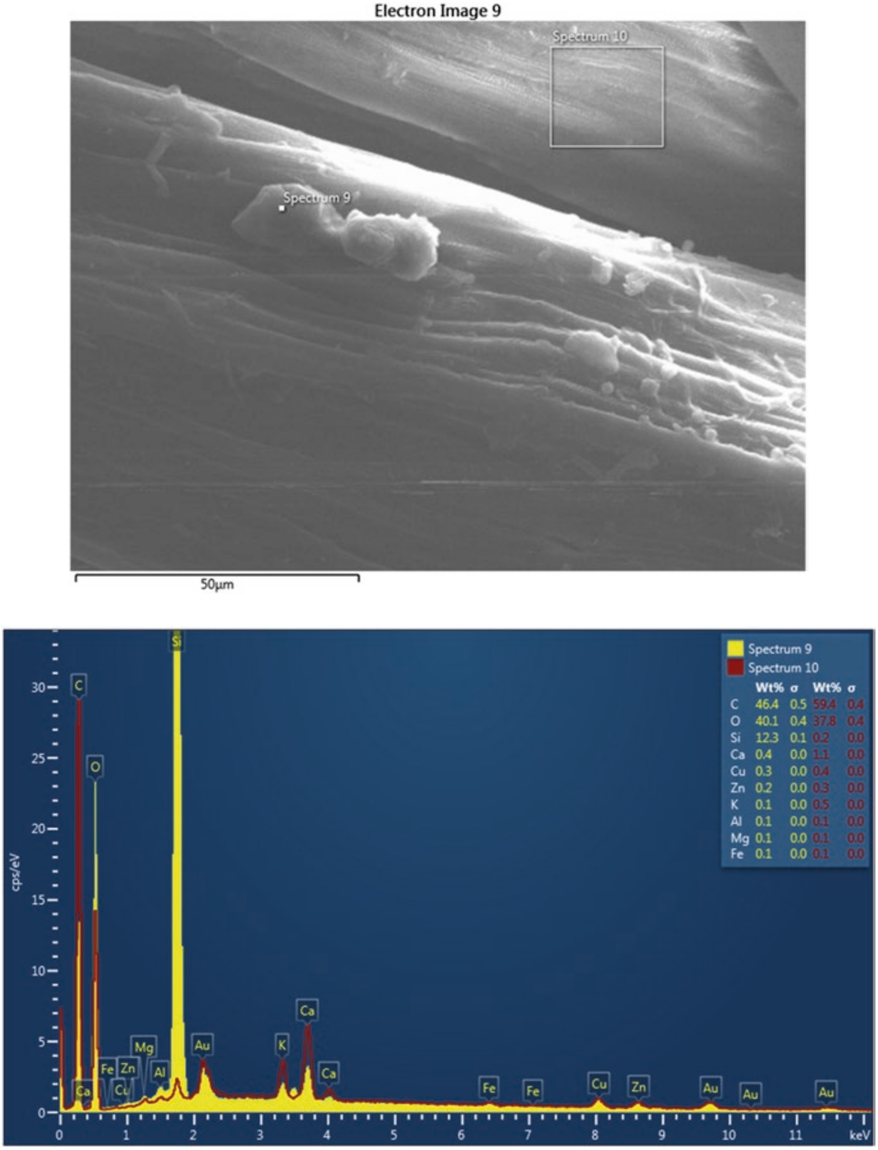
	Wt%	σ	Wt%	σ
O	50.4	0.4	43.8	0.3
C	37.5	0.5	55.0	0.3
Si	4.6	0.1	0.2	0.0
Al	2.0	0.0	0.1	0.0
Ca	2.0	0.0	0.4	0.0
Fe	1.4	0.0	0.0	0.0
Mg	1.0	0.0	0.1	0.0
K	0.5	0.0	0.1	0.0
Cu	0.4	0.0	0.2	0.0
Zn	0.3	0.0	0.2	0.0

Fig. 5.4 (**a**) Comparison of two spectra of the surface of *Hypnum cupressiforme* – control sample (1 mm). (**b**) Comparison of two spectra of the surface of *Hypnum cupressiforme* for the scan given in Fig. 5.4a



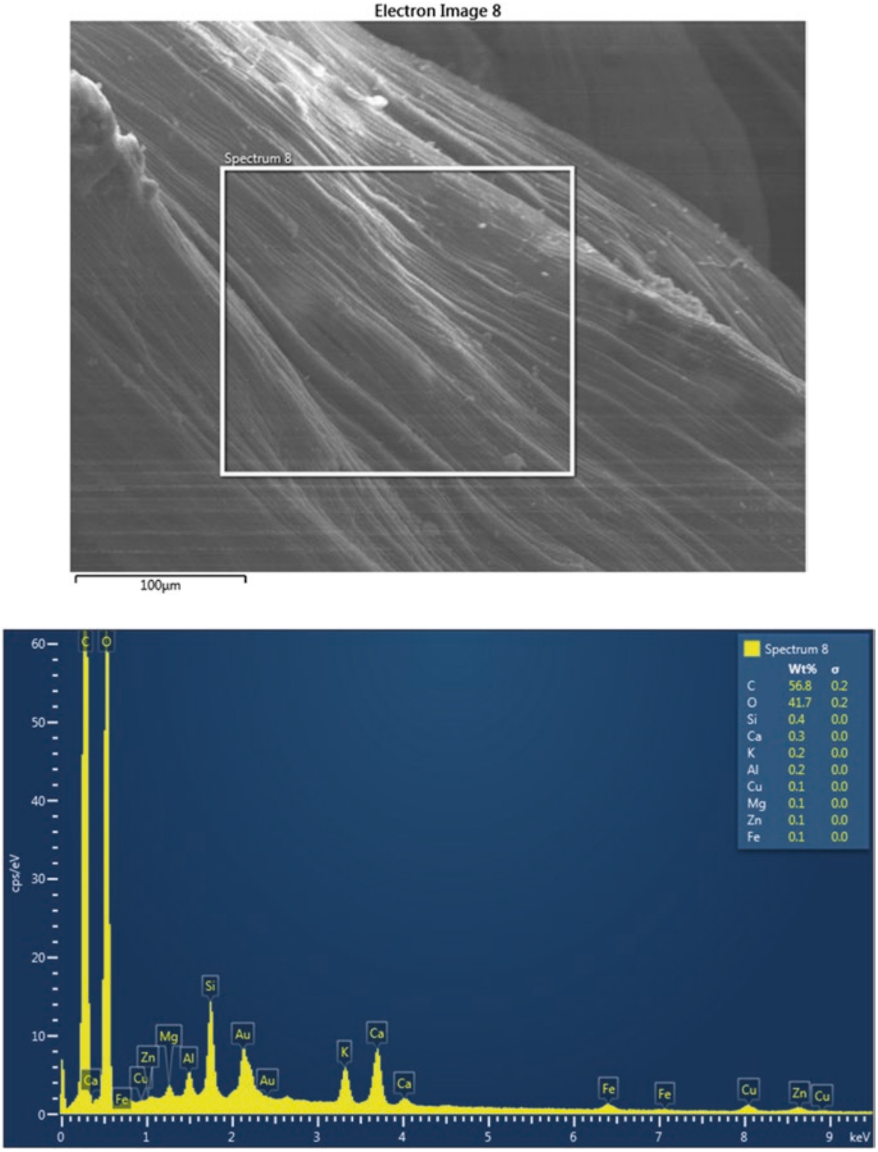
	Wt%	σ
C	58.0	0.3
O	40.2	0.3
Ca	0.7	0.0
Cu	0.3	0.0
K	0.2	0.0
Zn	0.2	0.0
Si	0.1	0.0
Mg	0.1	0.0
Al	0.1	0.0
Fe	0.0	0.0

Fig. 5.5 (**a**) Surface analysis of *Homalothecium lutescens* (25 µm) from a potentially polluted site. (**b**) Chemical composition of *Homalothecium lutescens* for the scan given in Fig. 5.5a



	Wt%	σ	Wt%	σ
C	46.4	0.5	59.4	0.4
O	40.1	0.4	37.8	0.4
Si	12.3	0.1	0.2	0.0
Ca	0.4	0.0	1.1	0.0
Cu	0.3	0.0	0.4	0.0
Zn	0.2	0.0	0.3	0.0
K	0.1	0.0	0.5	0.0
Al	0.1	0.0	0.1	0.0
Mg	0.1	0.0	0.1	0.0
Fe	0.1	0.0	0.1	0.0

Fig. 5.6 (**a**) Comparison of two spectra of the surface of *Homalothecium lutescens* (50 µm) from a potentially polluted site. (**b**) Comparison of two spectra of the surface of *Homalothecium lutescens* for the scan given in Fig. 5.6a



	Wt%	σ
C	56.8	0.2
O	41.7	0.2
Si	0.4	0.0
Ca	0.3	0.0
K	0.2	0.0
Al	0.2	0.0
Cu	0.1	0.0
Mg	0.1	0.0
Zn	0.1	0.0
Fe	0.1	0.0

Fig. 5.7 (**a**) Surface analysis of *Homalothecium lutescens* (100 μm) from a potentially polluted site. (**b**) Chemical composition of *Homalothecium lutescens* for the scan given in Fig. 5.7a

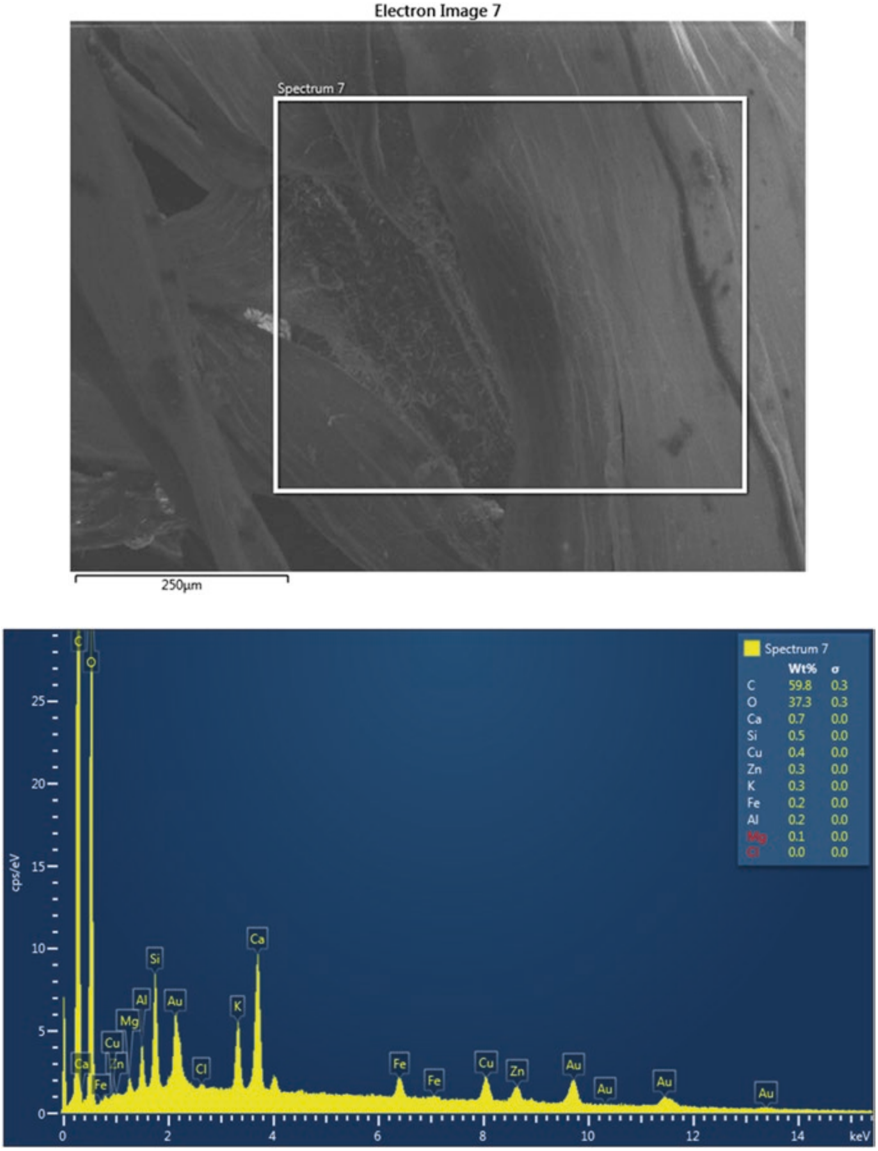


Fig. 5.8 (**a**) Surface analysis of *Homalothecium lutescens* (250 μm) from a potentially polluted site. (**b**) Chemical composition of *Homalothecium lutescens* for the scan given in Fig. 5.8a

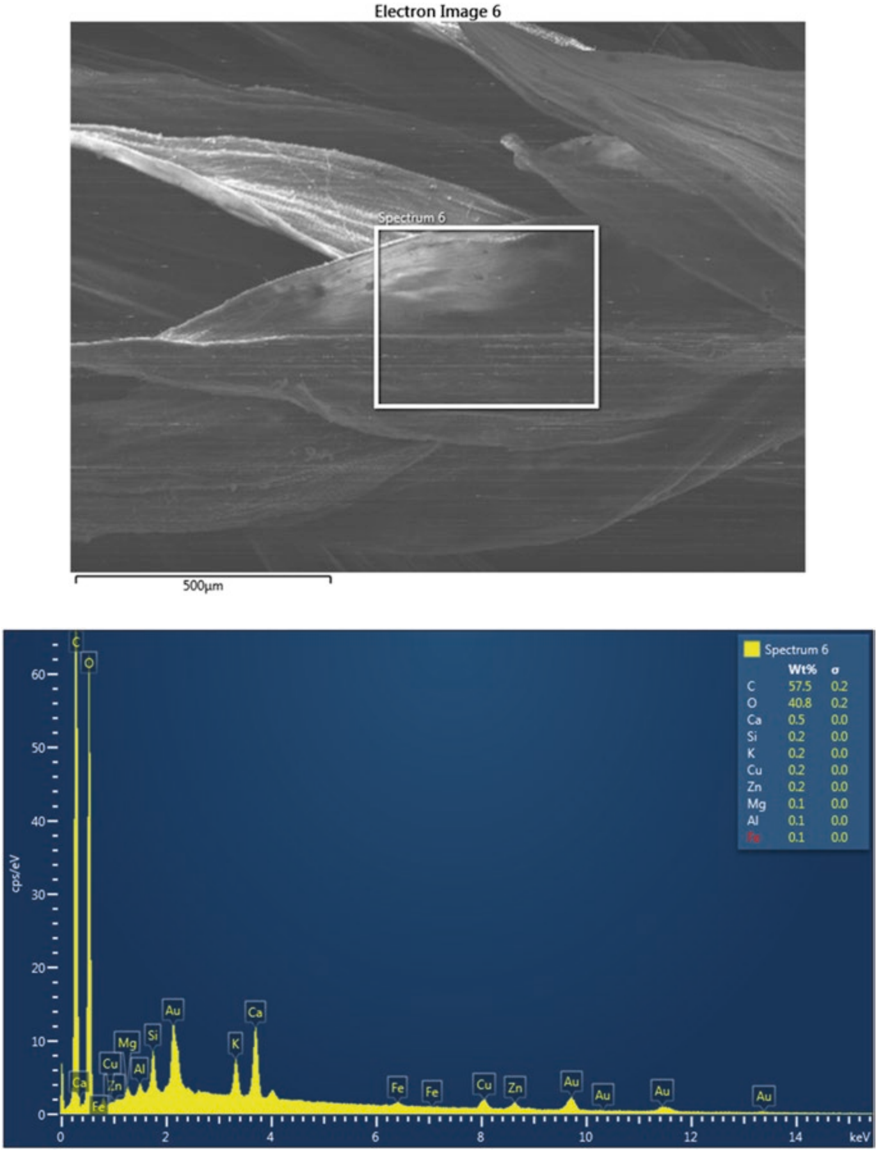
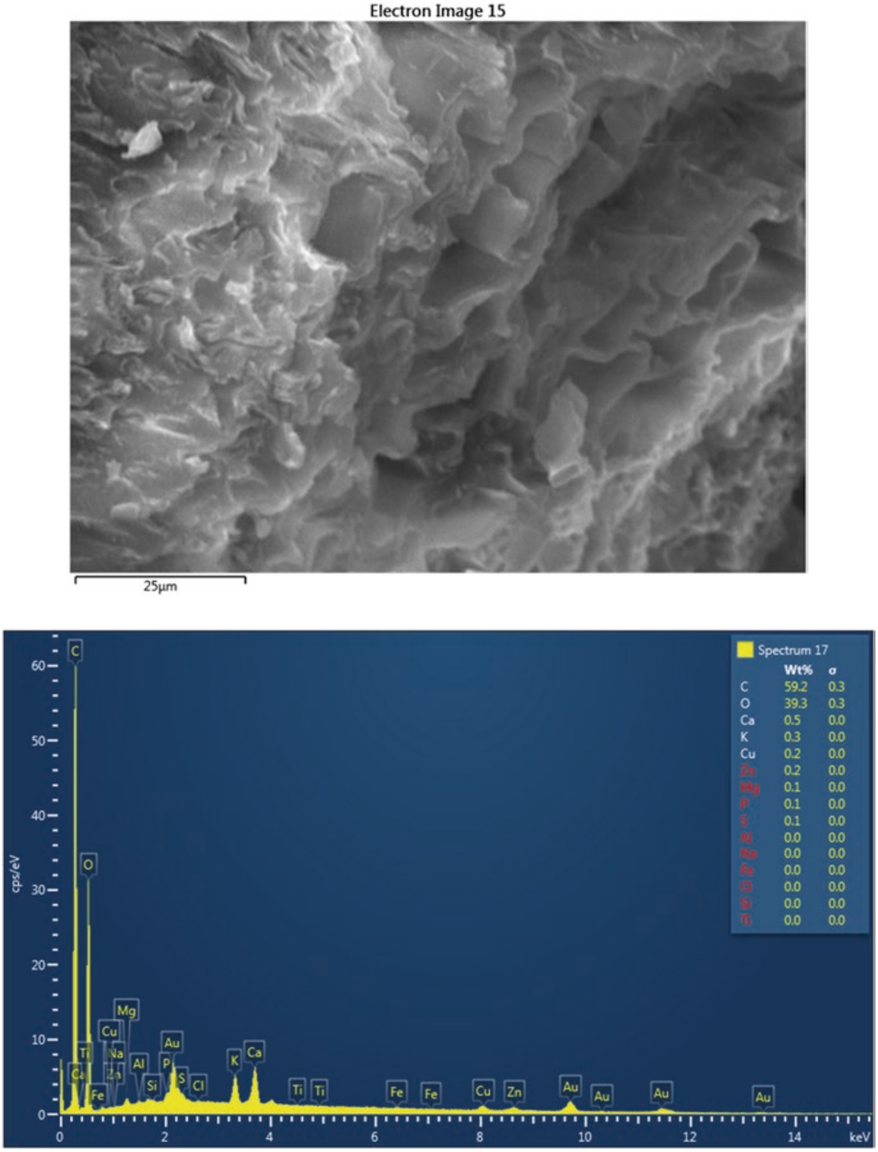


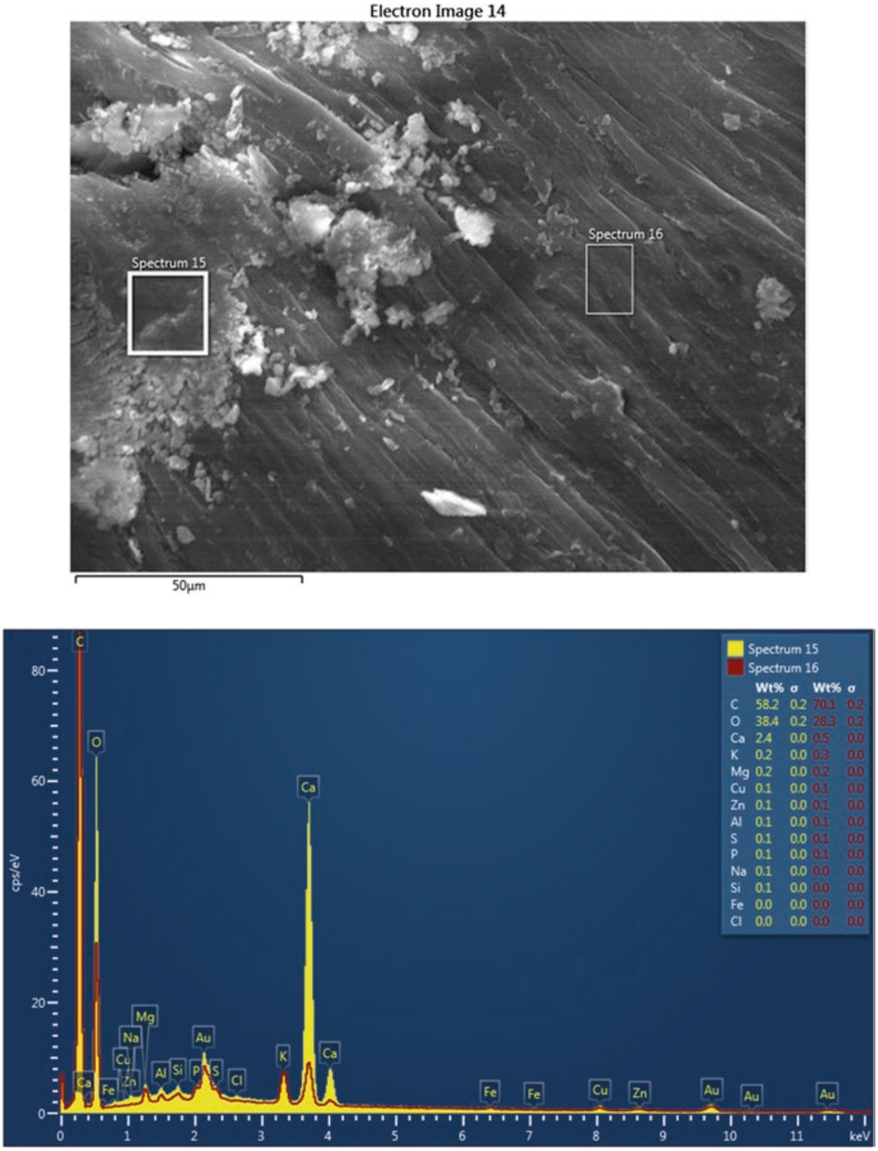
Fig. 5.9 (**a**) Surface analysis of *Homalothecium lutescens* (500 µm) from a potentially polluted site. (**b**) Chemical composition of *Homalothecium lutescens* for the scan given in Fig. 5.9a



Spectrum 17

	Wt%	σ
C	59.2	0.3
O	39.3	0.3
Ca	0.5	0.0
K	0.3	0.0
Cu	0.2	0.0
Zn	0.2	0.0
Mg	0.1	0.0
P	0.1	0.0
S	0.1	0.0
Al	0.0	0.0
Na	0.0	0.0
Fe	0.0	0.0
Cl	0.0	0.0
Si	0.0	0.0
Ti	0.0	0.0

Fig. 5.10 (**a**) Surface analysis of *Hypnum cupressiforme* (25 μm) from a potentially polluted site**.** (**b**) Chemical composition of *Hypnum cupressiforme* for the scan given in Fig. 5.10a



	🟨 Spectrum 15		🟥 Spectrum 16	
	Wt%	σ	Wt%	σ
C	58.2	0.2	70.1	0.2
O	38.4	0.2	28.3	0.2
Ca	2.4	0.0	0.5	0.0
K	0.2	0.0	0.3	0.0
Mg	0.2	0.0	0.2	0.0
Cu	0.1	0.0	0.1	0.0
Zn	0.1	0.0	0.1	0.0
Al	0.1	0.0	0.1	0.0
S	0.1	0.0	0.1	0.0
P	0.1	0.0	0.1	0.0
Na	0.1	0.0	0.0	0.0
Si	0.1	0.0	0.0	0.0
Fe	0.0	0.0	0.0	0.0
Cl	0.0	0.0	0.0	0.0

Fig. 5.11 (**a**) Comparison of two spectra of the surface of *Hypnum cupressiforme* (50 μm) from a potentially polluted site. (**b**) Comparison of two spectra (elemental composition) of the surface of *Hypnum cupressiforme* for the scan given in Fig. 5.11a

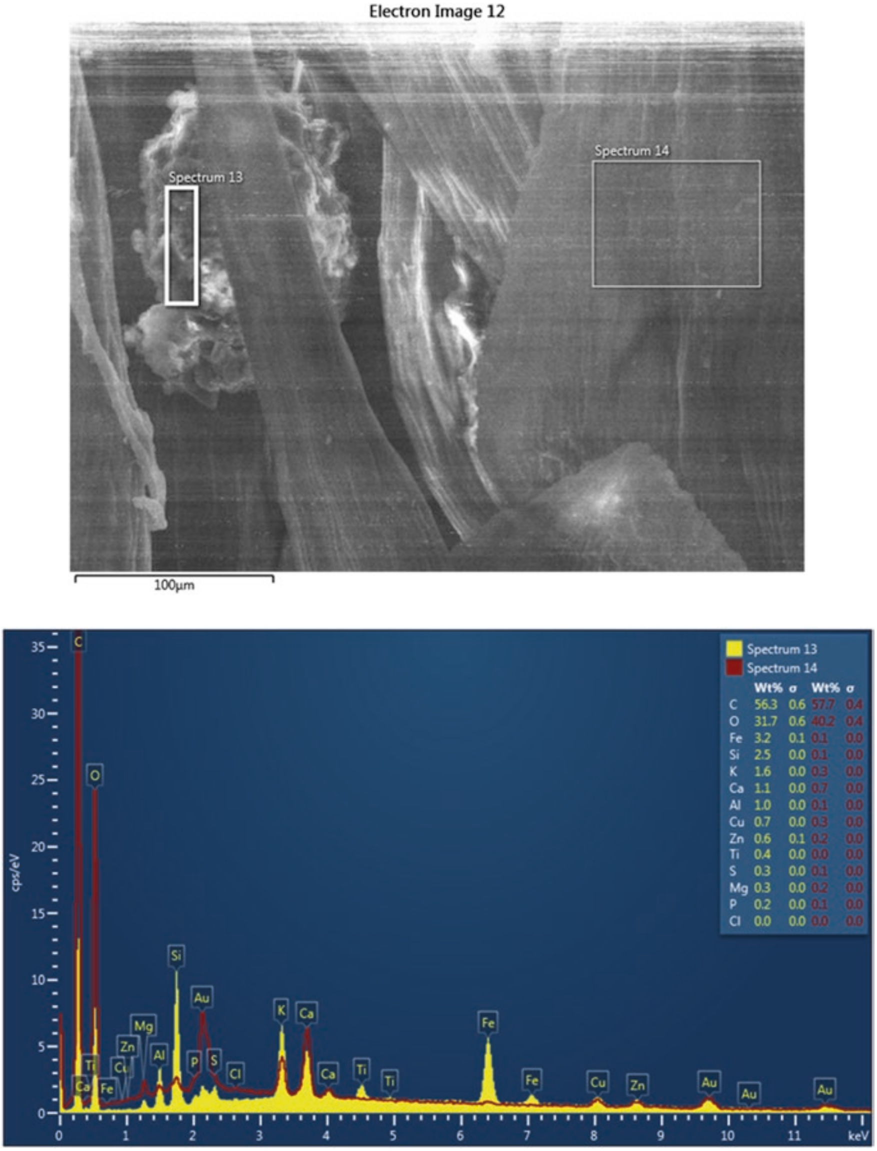


Fig. 5.12 (**a**) Comparison of two spectra of the surface of *Hypnum cupressiforme* (100 µm) from a potentially polluted site. (**b**) Comparison of two spectra (elemental composition) of the surface of *Hypnum cupressiforme* site for the scan given in Fig. 5.12a

elements that are not of interest. This occurs when the energy difference between the elements not of interest and elements of interest is equal to the energy resolution of the spectrometer. Figures below present X-ray mapping of both moss species. The resolution of X-ray mapping is limited by "analysis area." But even when a particle smaller than the analysis area is present on the specimen surface, sometimes this particle can be recognized. On the other hand, a method that quantitatively analyzes the specimen one by one while scanning the electron probe is called "quantitative mapping." Using this method, even if the P-B ratio is low, accurate distribution of elements can be obtained. This is an advantage of quantitative mapping, which cannot be achieved using simple X-ray mapping (qualitative mapping). Figures 5.13 and 5.14 present the mapping, which is used to color the image depending on the elementary composition. We choose different colors manual for each detected element. The duration time for this kind of analysis is a minimum of 15 min; longer period is better for better colorized image.

5.8 Conclusion

In the last decade, there has been a research expansion of methodologies that establish fast and effective methodological approaches for monitoring atmospheric deposits. Air pollution has been alarming in recent decades globally. The distribution of microparticles originating from different emission sources poses a huge

Fig. 5.13 (**a**) Mapping for elementary composition of *Hypnum cupressiforme* sample (**b**) Elementary composition using SEM of *Hypnum cupressiforme* sample

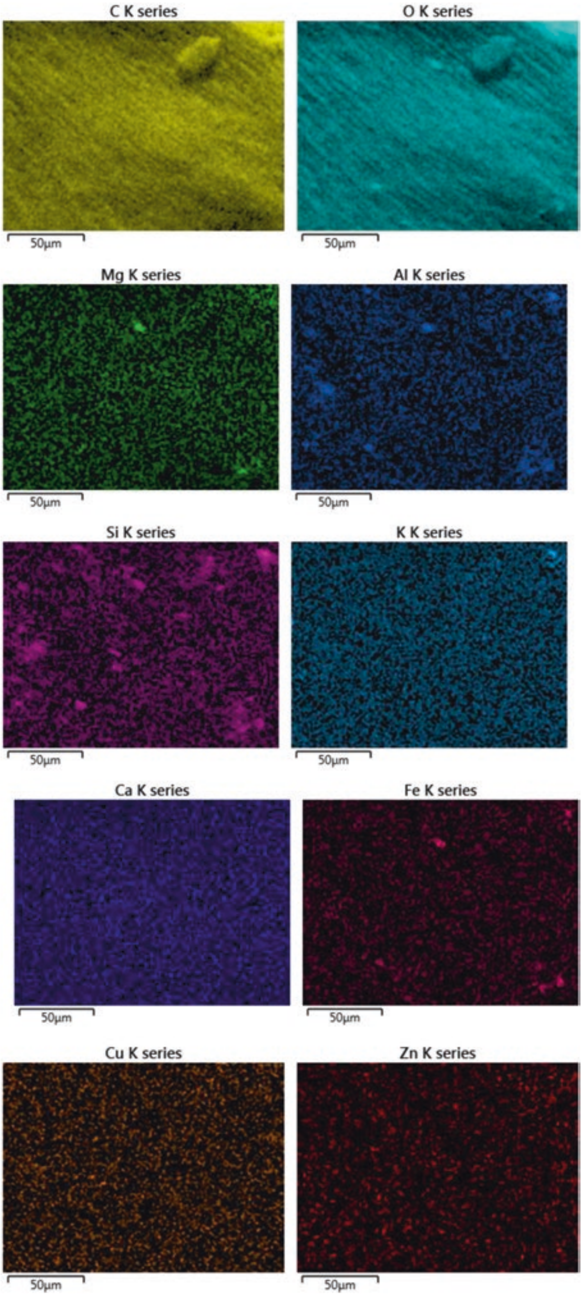


Fig. 5.13 (continued)

Fig. 5.14 (**a**) Mapping for elementary composition of *Homalothecium lutescens* sample. (**b**) Elementary composition using SEM of *Homalothecium lutescens* sample

threat to the health of the human population. The number of researches done so far is huge, but the most common problem is to find a fast, effective, and transparent research model. The proposed chemometric model can serve as an initial research for subsequent screening models. This chemometric model, unlike the typical analysis with existing chemical instrumentation techniques, does not require chemical destruction of the sample and additional costs for chemical agents. But on the other hand, this model is suitable for screening for large areas in order to identify critical areas. Quantitative chemical analysis still remains an irreplaceable identification method for determining the multi-element distribution in the environment. Bryophytes are a very useful tool for monitoring atmospheric deposition. This study also introduced the ability to monitor isolated atmospheric deposition on the surface of these plant species. Their unique structure and physiology allow dust particles to remain trapped on their surface. With the help of electron microscopy, the composition of anthropogenic elements in the dust particles trapped on the surface of these structures can be monitored. Chemical characterization is possible, but additional research is needed to compare analytical methods with these non-destructive analyses. Both types of moss, which have been proven to be effective bioindicators, were used in this study. But when examining large areas, it is necessary to use them alternately given their specific geographical distribution. Analyses have shown that they have similar surface adsorption to dust particles, as well as insignificant variability in the chemical composition plant surface: extracted for the biogenic elements carbon and oxygen; macroelements Mg, Al, Si, K, and Ca; and microelements

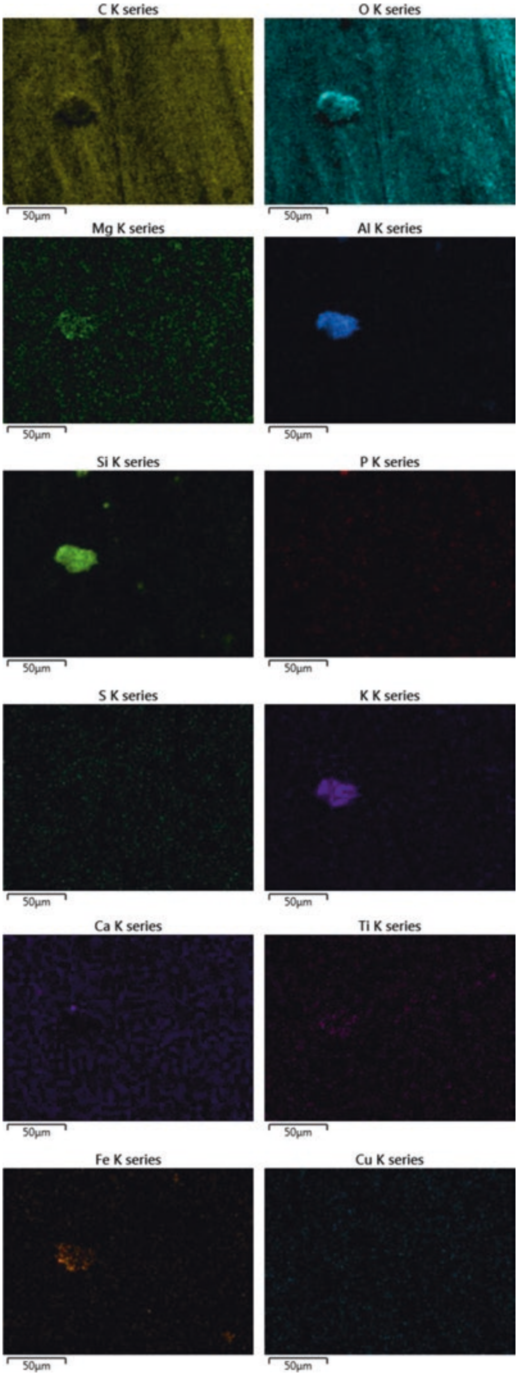


Fig. 5.14 (continued)

Fe, Cu, and Zn. Both moss species can be used interchangeable for dust deposition investigation.

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Chapter 6

Evidence for Atmospheric Depositions Using Attic Dust, Spatial Mapping and Multivariate Stats

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Abstract Atmospheric deposition poses significant ecological concerns. It is very important in air pollution researches to provide fast and efficient access to qualitative and quantitative characterization. Case study was introduced in order to implement the multidisciplinary approach in the investigations. When monitoring the distribution of certain substances in the air, it is necessary to choose very carefully the medium that will reflect not only the current but also the long-term atmospheric deposition. Attic dust was examined as historical archive of anthropogenic emissions, with the aim of elucidating the pathways of enrichments associated with exploitation of Cu, Pb and Zn minerals in the Bregalnica river basin region. Attic dust samples were collected from 84 settlements. At each location for attic dust sampling, topsoil samples from the house yards were also collected. Mass spectrometry with inductively coupled plasma (ICP-MS) was applied as an analytical technique for multi-element determination. The universal kriging method with linear variogram interpolation was applied for the construction of spatial distribution maps. Data interpretation was considered in correlation with dominant geological units. Significantly enriched contents for Cd, Cu, Pb and Zn have been correlated with the lithogenic dominance of Rifeous shales. Both Pb-Zn mine environs were identified as the most affected areas with lead and zinc enrichments, due to the

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continually long-time wind dust dispersion from the flotation tailings. Atypical deposition was revealed for In, Te and W, as *silent* tracker for air pollution.

Keywords Air pollution · Attic dust · Kriging modelling · Mass spectrometry

6.1 Introduction

The biosphere is the factor that develops the natural environment. It covers the surface parts of the lithosphere, the lower part of the atmosphere and the hydrosphere. It is a complex system in which numerous biological and physical-chemical processes and reactions take place. The natural balance of these processes is most often disturbed by the action of the anthropogenic factor. Because the relative homeostatic balance in the environment is necessary for survival and survival of the living world, research into the chemical composition of air, water and soil is imposed as an operative. In certain situations where the human activity is the predominant factor, certain degrading processes occur in the soil. During the excavation of geological minerals, the natural balance of most of the chemical elements that in some forms are incorporated into the minerals can be displaced (Sengupta 1993; Salomons 1995). As a result of these activities, there is an absolute imbalance in the environment, thereby shifting the normal habitat conditions of the organisms. As a consequence of this condition, ecological balance in the environment may be endangered. VanLoon and Duffy (2000) term these conditions as *specific environment*. One of the most significant shifts in the natural balance of the environment is the heavy metals that can be deposited in higher areas in certain areas. In this case, toxic conditions are created in certain parts of the environment. The most sensitive to changes in the content of heavy metals is of course the surface layer of the lithosphere. Soils are not the only medium that is threatened and affected by anthropogenic activities. The entire biosphere is affected by changes in the natural distribution of some metals, most notably heavy metals and some toxic semimetals (Athar and Vohora 1995; Siegel 2002; Artiola et al. 2004; Salminen et al. 2005; Duruibe et al. 2007). Excavation of mineral raw materials is most often accompanied by a shift in the natural balance of chemical elements in the soil. This situation is due primarily to the displacement of the basic regolith material, on the basis of which the surface soil is formed. Such destructive changes in the surface of the lithosphere disrupt the natural distribution of the minerals. But on the other hand, these processes also lead to the release of a large amount of dust that is distributed to the environment. The chemical composition of the particles in this dispersed ring is usually high in some heavy metals. Mining and flotation tailings are also subject to the same process, which are deposited in the immediate vicinity of the mines. This mine and flotation waste can be washed by wind as well as rains (Van het Bolcher et al. 2006; Šajn 2006).

6.2 Anthropogenic Effects on Atmospheric Emissions

The emission of toxic substances into the atmosphere is one of the greatest health threats to the light. Lice are directly exposed to the effects of harmful substances through inhalation. Particle gases depend on their size and on the defence capabilities of the respiratory tract. Exposure to toxic substances is usually defined as a function of their concentration and time of exposure, i.e. the phenomenon that occurs with the achievement of direct contact between humans and the environment with the content of pollutants in a given interval of time. The health effects of toxic substances depend on their concentration and the place of their deposition in the respiratory tract. The most at-risk group in the population of harmful effects of airborne particles are children and the elderly, especially those with a weakened cardiovascular and respiratory system. Heavy metal air pollution is a global process that affects every part of the Earth. Rapid increases in the concentrations of pollutants in the atmosphere and the environment are most often associated with the development of technologies for exploitation and processing of mineral raw materials. This change exposes the biosphere to the risk of destabilization of organisms that grow under conditions with low concentrations of these substances and that do not have developed biochemical pathways capable of their detoxification when they are present in high concentrations (Hoenig 2001).

Atmospheric deposition of particles containing potentially toxic metals ("heavy metals") is the main subject of many studies and usually occurs in industrial areas, in places where natural resources are exploited and processed as well as in areas with large settlements where which heating, traffic and municipal waste are the main sources of pollution (Wong et al. 2006). Heavy metals in the atmosphere originate mainly from the dispersion of dust from metal refining, combustion of fossil fuels and other human activities and remain in the atmosphere until they are removed by various cleaning processes. Substances released into the atmosphere through the combustion process can be transported to places away from wind sources, depending on whether they are in gaseous or solid form. Urban pollution with heavy metals has been the subject of many studies (De Miguel et al. 1999; Ajmone-Marsan et al. 2008; Beelen et al. 2009). Regional soil contamination occurs mainly in industrial regions and in the centres of large settlements, where factories, traffic and municipal waste are the most important sources of environmental pollution. Due to the heterogeneity and the constant change of the urban environment, it is necessary to first determine the natural distribution and the methods for distinguishing artificial anomalies in nature. The natural background itself is variable, which means that higher concentrations of some substances may be normal for one region but abnormal for others. However, there are cases when some industrial processes, especially mining and metallurgical, when located near cities, can lead to an increase in environmental pollution (Ajmone-Marsan et al. 2008).

6.3 General Meaning of the Term "Dust"

The term "dust" does not have a precise meaning, nor is it defined definitively as a premature solid and a dusty particle. When determining hazards, particle size is just as important as the nature of the dust. In general, the most dangerous types of dust are with very small particles that are not visible to the human eye, as is the case with fine powders. These types of particles are small enough to be inhaled, but at the same time they are large enough to stop in the lung tissue and not be able to exhale. However, some substances create a very coarse dust of large particles, which can also be dangerous. Substances can form dusts with particles of different sizes – the fact that you can only see large dust particles does not mean that small particles are not present.

There are several basic types of dust that are released and distributed in the environment:

- Nanomaterials: Nanomaterials are used in many modern processes. They are especially dangerous because they can be absorbed directly into the bloodstream by inhalation, through the skin and lung membranes. They should be considered hazardous to health no matter what material they are made of. Conventional protective equipment will not provide adequate protection, so you must consult your laboratory before opening or intentionally sampling such products.
- Toxic dusts: Toxic dusts are usually formed when working with substances that are themselves toxic (e.g. lead, mercury, chromium, etc.). Inhalation can damage the lungs, or they can spread throughout the body by entering the bloodstream.
- Harmful dusts: Harmful dusts may occur when handling materials such as flour/cereals, resin, tobacco, sugar, paper, dry food, cement, sawdust, coffee beans and tea and black ink (as in toner in a photocopier/printer). These types of dust are usually only irritating, but in concentrated form, they can be dangerous to health. Hard (deciduous) wood dust is carcinogenic.
- Flames of the flame: Flames of the flame are prone to flare and flare and the flame can be exposed to fire or explosion. Njihovo zapaljenje moie biti izazvano iskrom ili otvorenim plamenom pa čak i slijeganjem na vruću površinu. When it comes to spraying, it is easy to get stuck, it can burn and burns, or it just burns – if you do not open the source of the spark. If there is an explosion in the back of the head, it is possible to increase the risk of leakage, which increases the risk of exposure.

Harmful dust can be found almost everywhere. Some of the most common are paper dust in mail sorting offices and paper mills, cement dust on construction sites and various types of dust in ports, in inland customs warehouses and in the entrepreneur's business premises and rooms with photocopiers/printers. Toxic or flammable dusts in dangerous concentrations can also be encountered at places of loading, unloading or trans-shipment of bulk cargo (e.g. cereals, metal ores, coal, etc.).

Dusts generally damage the lungs and respiratory system, but some species can also cause cancer. The major diseases associated with inhalation of hazardous dusts are:

- Benign pneumoconiosis. Disease caused by inhalation of harmless dust that accumulates in the lungs to such an extent that they are visible on an X-ray. They do not damage the lung tissue, and therefore the disease does not create difficulties. This condition is most commonly associated with metal dusts such as iron and tin.
- Pneumoconiosis. Common name for a group of chronic lung diseases caused by inhalation of ore dust particles. The term includes several diseases that are named after the dust they are caused by. The most famous are asbestosis (from asbestos dust), silicosis (from dust containing silicates) and talc (from talc dust).
- Pneumonitis. Inflammation of the lung tissue or bronchioles, mainly caused by inhalation of certain metal dusts. The symptoms are reminiscent of pneumonia, but vary in severity, depending on the metal inhaled. It is most often caused by cadmium and beryllium dusts.
- Lung mesothelioma. Lung tumour, mainly caused by asbestos and toxic metals exposure.
- Lung cancer. It can also be caused by exposure to asbestos and heavy metals.

6.4 Dust Emissions and Distribution

The physical properties of dust obey natural laws that are not identical to those that apply to solid and liquid bodies. Thus, for example, the pressure on the walls of a box full of dust does not have to be the same at all points. Dust is harmful for several reasons, because it can cause health problems not only for workers who have daily contact with it but also for the population in the wider environment. Erosions that occur in landfills under the influence of wind are one of the main sources of dust. Landfill work itself also leads to dust emissions. Landfill management is a complex process and often requires constant traffic to and around the landfill where dust emissions also occur.

The intensity of dust release depends on several factors: characteristics of coal, moisture content, particle shape, landfill geometry, position and method of formation and climatic conditions. The determination of the dust generated depends on one key factor and that is the amount of moisture. The moisture that is in the landfills is a consequence of precipitation, the process of washing coal, etc. Climatic conditions affect the amount of dust emission. Regular control of dust emissions at landfills is important because of environmental protection and economy.

The most favourable moment for the selection of systems that serve for protection against dust is in the landfill planning phase. In the end, this turned out to be a much more cost-effective way than taking action afterwards. The basic methods of dust control are wind control, landfill coverings, wetting dust emission control systems and by fencing the landfill. The dust that collects in the house is a combination of atmospheric dust and dust of human origin, which consists mainly of extinct skin cells and fibres from clothes and blankets. It can be removed with a broom, cloth or vacuum cleaner. Mites, organisms that are often found in carpet fibres and on mattresses, use organic components of house dust for their diet. Landfill dust levels can lead to dust hazard zones. "Monitoring" dust levels can identify dust endangered areas. Certainly, successful airborne dust monitoring requires a good choice of menu locations (Finlayson-Pitts and Pitts 1999).

6.5 Natural Archives of Distributed Dust

Attic dust is defined as dust that accumulates on wooden carpentry of attics, the spaces in which the influence of inhabitants is minimized. Attic dust is derived predominantly from external sources through aerosol deposition and as a result of soil dusting (Davis and Gulson 2005). There is a little influence from household activities (Šajn 2003). Its mineralogy includes phyllosilicates (montmorillonite and illite) and calcite and dolomite in areas of outcropping carbonate rocks (Šajn 2000). The deposition of attic dust seems to be continuous through time in undisturbed attics. Its chemistry, therefore, reflects the average historical levels of the atmospheric pollution. In previous geochemical studies (Šajn 2002, 2003, 2005), the properties of attic dust in Slovenia (regional-scale) were established. Attic dust was also successfully applied in tracing a plutonium halo, a result of atomic bomb experiments, in Nevada (Cizdziell et al. 1998; Coronas et al. 2013). Close to each sampling location, an old house with intact attic carpentry was chosen. Most of the selected houses were at least 100 years old. To avoid collecting particles of tiles, wood and other construction materials, the attic dust samples were brushed from parts of wooden constructions that were not in immediate contact with roof tiles or floors.

The determination of historical emissions is based on the data of heavy metal concentration in the attic dust from different measurement sites of the weight of total monthly air deposit (Lioy et al. 2002; Jemec Auflič and Šajn 2007; Žibret and Šajn 2008; Žibret 2008). The main idea behind determining past emissions is that heavy metal characterization of deposited dust on a small area is multiplied by the concentration of the elements in that area; the mass of the polluter which has been transported to the place of interest by air can be determinate (Šajn 2006; Žibret 2008; Žibret and Šajn 2008). Undisturbed attic dust is potential archive for atmospherically deposited particles and has been shown to be effective across urban areas and in the vicinity of smelters, mines and other potentially emission sources (Cizdziell et al. 1998; Cizdziel and Hodge 2000; Šajn 2000, 2001, 2002, 2003, 2005, 2006; Alijagić 2008; Alijagić and Šajn 2011; Völgyesi et al. 2014; Angelovska et al. 2016; Balabanova et al. 2019). Atmospheric emissions attributed to the extraction stage of mining come mainly from the action of wind on disturbed land and stockpiles of ore and waste material. As a result of these processes, dust is permanently introduced in the atmosphere (Ilacqua et al. 2003). Usefulness of attic dust as a suitable long-term monitor for the determination of the status and content in air is proven by numerous studies. Respective analytical work was first presented by Cizdziell et al. (1998), analysing ^{137}Cs and ^{239}Pu near the Nevada nuclear test site. Similar application was conducted across the past 20 years, *hunting* the anthropogenic emissions sources and their impact to the environment (Cizdziel and Hodge 2000; Ilacqua et al. 2003; Gosar et al. 2006; Gosar and Teršič 2012; Tye et al. 2006; Hensley et al. 2007; Balabanova et al. 2011; Bačeva et al. 2012; Coronas et al. 2013; Pavilonis et al. 2015; Balabanova et al. 2019). Despite the metal's enrichment catchment, attic dust should be also considered as a poly-metallic marker for the lithogenic distribution of elements. De Miguel et al. (1999) have proposed a dust model for tracking the natural phenomena of poly-metallic geochemical association in urban areas. However, other investigations emphasize only the anthropogenic enrichments (Fordyce et al. 2005; Goodarzi 2006; Tye et al. 2006; Balabanova et al. 2011, 2019).

6.5.1 Collecting Attic Dust Protocol

Attic dust represents the long-term (historical) deposition of distributed dust in the air. This medium, which is created for a long time, is a natural archive of particles that are distributed for a long time in the lower layers of the atmosphere and then under certain conditions are deposited in a certain environment. In this way, the particles that bind various substances (gases, liquids and solids) are collected and thus can give a clear picture of the exposure of organisms to certain pollutants that are distributed in the air. When setting the protocol for collecting this type of sample (i.e. medium) through which the monitoring of the pollution conditions in the environment is performed, one must pay attention to the age of the houses where this dust is collected. There is still a habit among the population not to remove dust from the attic beams for a long time. Before the dust collection process, it is necessary to conduct a survey with the population to determine the age of the house (year of construction) as well as the habits of the people who live there. This is necessary in order to determine for how long the dust has been deposited in a certain place (location) so that the results of the chemical analysis of these samples can be correctly interpreted for a certain period of time.

The process of collecting a single sample needs to be performed very carefully. First, a precisely defined protocol should be set (prepared) for locations from where the samples should be collected. The researcher determines the size of the space around the emission source or the area to be covered by the monitoring. Then the field activities begin, where it is necessary to find houses/dwellings that were built before the emission occurs (in the case of anthropogenic emission or natural disaster, which occurred in a certain period).

The dust sample is collected from the surface of the attic beams. First, the visible dirt that has accumulated on the attic beam is removed, and then the finest dust is carefully collected from the surface with the tip of a polyethylene brush. At least ten

Fig. 6.1 Collecting protocol for attic dust

places are selected from where sub-samples are collected to prepare a representative sample from one location (house). Each sample is collected in plastic bags and delivered to a laboratory for physical-chemical preparation and chemical analysis (Fig. 6.1).

6.6 Data Mapping and Interpretation

The main purpose of such research is to visualize the distribution of certain substances in the air and to determine places of deposit for a longer period of time. The most commonly used model for visualization is the kriging method (Wellmer 1998). This is the methodology that was introduced as a geostatistical model. Classical statistical methods are based on the assumption that individual samples (e.g. sample value at drilled holes or pipes) are statistically independent of each other. This condition is satisfied on the banal example of rolling the dice. If a six is thrown, it means that the chance of her turning again in the next roll is equal to the chance of turning every other number. The chance of turning is 1/6 because "the chance has no memory". This type of independence can rarely be applied to mineral deposit (ore) data. Every geologist knows that the chance of digging a hole of a higher class is higher at the site of a previous site of a higher class than at a site of a lower class. There is therefore a certain spatial interdependence. Geostatistics is statistics in which this spatial association is taken into account; there are variables known as regionalized variables (de Smith et al. 2009). We will deal here only with those calculations that can be performed either manually or by diagram. There are two areas where geostatistical calculations can be important, even in the early stages of ore assessment: calculation of errors or uncertainties in reserve estimates and therefore the possibility of classifying assets and reserves and determination of the class for excavated blocks, especially if the application of the class results from individual blocks of ores or untreated finds in ore deposits (de Smith et al. 2009).

6.6.1 Digital Field Modelling and Possible 3D Views

Digital terrain modelling aims to form a mathematical model that will faithfully represent the surface of the terrain and enable various analyses and applications (de Smith et al. 2009). In order for these analyses to be performed efficiently, bearing in mind that 3D models usually consist of a large amount of data, special organization and standardization of data is required. In essence, the process of forming a 3D model consists of selecting and implementing a data structure and an appropriate creation method. The surface of the terrain is usually represented by a set of points and lines arranged on the surface of the terrain in an appropriate manner and arranged in the necessary structure for easier handling of this data. Also, an integral part of the 3D model are the methods by which, in addition to the given data structure, the topographic surface is defined in the geometric and geomorphological sense. In general, the surface of the terrain can be presented in three ways, as follows:

- Isohypses
- Through the functions of two variables
- Volumetric (volume) model

The first way, i.e. the representation of the terrain with isohypses, is the cross section of the terrain surface and horizontal planes placed at appropriate heights. This section is a curved line that we call isohypses. This is the most commonly used way when it comes to presenting terrain on the cartographic bases. This way of presenting the terrain surface is characterized by high quality in the geomorphological sense, because all the important relief characteristics of the terrain are included in this way. Also, when the terrain is mathematically represented by isohypses in digital form, the surface of the terrain is not given explicitly, but it is given implicitly over the cross section of that surface with horizontal planes. That's why this way modelling the terrain surface is not an exact enough method, because the question arises as to what happens to the values of the terrain height between two adjacent isohypses. This dilemma is especially pronounced in places where characteristic relief forms appear, such as peaks, bottoms, watersheds, watersheds, valleys, etc.

Another way to represent the terrain surface in digital form is to use the function of two variables, where those variables belong to the corresponding domain. Most often, these are functions in which a unique location value is obtained for a given location (usually planimetric coordinates x and y of the local coordinate system, state coordinate system or even geographical coordinates). In this case, it is a 2.5D (2D + 1D) model.

Terrain models that enable the representation of surfaces where one height can be obtained for one x, y location, i.e. surfaces for which the surface function $f(x, y)$ has a value in the form of a vector, are called 3D models. In these models, all three coordinates are completely equal. The most well-known and in practice the most common 3D terrain models based on these principles are digital terrain models

(DMT), whose basic data structure is in the form of grid and TIN (triangular irregular network) (Li et al. 2005).

And the third way to represent the surface of the terrain is to use a volumetric model, where the objects of space are represented by volumetric elements. One of the typical examples is the use of voxels (volume elements, most often cubes or prisms) of sufficiently small dimensions. This is analogous to the representation of phenomena in digital raster images (or raster GIS), with the difference that digital images are 2D displays. Namely, in these models, the terrain is presented as a body composed of a set of interconnected voxels. Using this data model, tunnels, caves, buildings and structures, settlements, etc. can be presented very easily. Another type of volumetric models are models based on the representation of bodies in space using the division of space into non-overlapping tetrahedra. This is analogous to using TIN to represent phenomena in 2.5D models with functions of two variables. Finally, it should be said that volumetric models are mainly represented in the presentation of infrastructure facilities and settlements, especially communications and traffic. They are also used in geophysics, but are very rarely used for modelling terrain surfaces (Li et al. 2005).

6.6.1.1 Digital Terrain Modelling in Grid Form

Digital terrain modelling in the form of a grid implies the methodology and technology of displaying the terrain over a set of points with known heights arranged in a regular grid – a grid. In this way, the surface of the terrain is actually represented by a 3D model called the digital elevation model (DEM) (Li et al. 2005). The advantage of digital terrain modelling in the form of grid is reflected in:

- Application of simple algorithms for handling and analysis of height models
- Savings in terms of archiving, i.e. only the heights of points (pixels) are stored, while their planimetric coordinates are implicitly given through the position of the initial network element, network orientation in space, dimensions and position of the observed network element in the height matrix
- Use of algorithms, software tools and formats for archiving and data exchange that are standardly used for raster GIS analysis and digital image processing
- Realization of simple algebraic operations on grid elements, the combination of which can perform very complex analyses, including advanced data compression and record pyramids for representing the terrain in other (different) resolutions

6.6.1.2 Digital Terrain Modelling in the Form of TIN

Digital data modelling in the form of TIN is represented in many practical solutions and software tools for the formation and analysis of 3D terrain models. When modelling terrain in the form of TIN, the vertices (nodes) of triangles are points with known heights (Li et al. 2005). The triangles are interconnected so that they better approximate the surface of the terrain being modelled. We distinguish between 2D and 3D triangulation. In 2D triangulation, the area covering the input data set in the XoY plane is divided into non-overlapping triangles. In other words, the triangulation of a set of points is a system of triangles whose vertices form a corresponding set, i.e. whose interiors do not intersect with each other and whose union completely covers the surface of the terrain. At the same time, using the values of heights at given points of the grid, spatial triangles are obtained that approximate the surface of the terrain (Li et al. 2005).

Also, in order to better approximate the relief, the terrain model is represented by triangular surface patches. This means, in addition to the height of the vertices of the TIN triangles, additional conditions relate to the values of the normal to the terrain surface at the TIN nodes and the conditions that require minimization of the curvature of the terrain surface. There are a number of criteria and algorithms for forming 2D TIN. The 2D Delaunay triangulation, which has particularly interesting geometric properties, is most commonly formed. Namely, Delaunay's triangulation maximizes the minimum angle of the triangles of the grid, i.e. it eliminates "elongated" triangles of the network. Also, in practice, for modelling the terrain, modifications of Delaunay's triangulation are often used in order to achieve some specific requirements in order to obtain the most accurate representation of the terrain surface. In some cases, it is required to install mandatory lines and polygons (structural characteristics of the terrain) in the model itself, so that these lines must be represented by the sides of the triangle TIN (Li et al. 2005).

When it comes to 3D triangulation, it consists of spatial triangles whose projections in the XoY plane can generally intersect, i.e. overlap. As in the case of 2D triangulation, Delaunay's tetrahedralization or 3D Delaunay's triangulation is most often used here. The data structure that represents the TIN can be based on storing data on the edges of triangles (triangle edges) or on storing data on the triangles themselves. In other words, in addition to the table containing the points (nodes) of the TIN, it is necessary to keep the table with the sides of the triangles for the first approach or the table with the triangles of the TIN for the second approach. In both cases, information that is not stored directly (data on triangles, i.e. on the sides of the network) can be obtained based on the network topology. In order to preserve information related to points, lines and surfaces that may be important for modelling the terrain surface, for TIN elements (nodes, sides and triangles), it is necessary, in addition to their geometry and topology, to keep the appropriate attributes, i.e. heights and mutual relations of the mentioned TIN elements (Li et al. 2005).

6.6.2 Interpolation Methods and Their Basic Features

The term interpolation comes from the Latin word "inter" which means between and the Greek word "polos" which refers to a point or a node. In other words, interpolation is defined as the process of determining a new (unknown) value between two or more known values of a function. The function in this case may be known, but in a complex form for computation. Also, the function may be unknown, but some other information about it is known, i.e. function values on a given set of points. It is this second case that is common in solving engineering problems and scientific and technical tasks. For example, when measurements yield only a certain number of function values, the so-called discrete set of points, it is necessary to determine the approximate values of a given function at other points. Estimation, i.e. interpolation, can be performed in one, two or three dimensions. The estimation can be performed on the basis of known values of the observed primary variable (autocorrelation) or with the help of the values of one or more other secondary variables in the same area. The condition is that the secondary variables are strongly correlated with the primary variable. Also, there are several methods that include bilinear and bicubic interpolation in two dimensions and trilinear interpolation in three dimensions (Li et al. 2005; de Smith et al. 2009).

Many interpolation procedures and methods are used in various fields of science and research. All these methods, i.e. interpolators, can be divided into several categories:

- Global/local interpolators
- Exact/approximate (approximate) interpolators
- Continuous (gradual, gradual)/intermittent (unconnected, sharp) interpolators
- Stochastic/deterministic interpolators

Global interpolators define a single function that determines values for the entire interpolation area. The change in one of the entered values is reflected in the total display area or interpolation area. Local interpolators use an algorithm that repeats the values of a smaller set of points relative to the whole set of point values. A change in one of the entered values only affects the results within the local area. Exact interpolators treat all points equally with which the interpolation is entered. Namely, the interpolation surface passes through all points whose values are known, i.e. advance date. Approximation interpolators are applied when there are indeterminate or unknown values of a given surface. They are applied in data sets where there are global trends that vary slowly, overshadowed by local fluctuations, and that vary sharply and produce certain errors (deviations) in given values. For these reasons, the smoothing effect reduces the effect of defects on the obtained (interpolated) surface. Gradual interpolators are methods with sufficiently small elements that are interconnected in continuity and that deviate minimally from given points. A typical example of gradual interpolators is the moving surface method (de Smith et al. 2009).

Intermittent approach methods involve sharp transitions and relatively rough barriers in the interpolation process. Interpolators (methods) are based on the concept of random variables. The interpolation surface is projected (imagined) as one of many that have been observed, that is, which can be obtained on the basis of known points with high probability. When it comes to deterministic methods, it should be borne in mind that they do not use probability theory and statistics or models of random processes (de Smith et al. 2009).

Unlike the classical statistical approach, geostatistics takes into account the spatial dependence of variables. Namely, geostatistical methods of interpolation start from the assumption that by knowing the value of a property at known points, it is possible to establish its value at unknown points as well. Assuming that the samples are representative and consistent, the values of the corresponding variable at a new salt location can be obtained using the appropriate interpolation method.

Interpolation methods include a set of procedures for creating assumed values of interest. The methods that can be applied to create a 3D model are:

- Finite element methods
- Interpolation methods using moving surfaces (interpolation with local polynomials, inverse distance method, oblique plane method)
- Methods with variational approach (minimum curvature method, spline method)
- Geostatistical methods (collocation method, linear prediction by least squares method, kriging method)
- Methods of forming TIN models based on input data and interpolation of heights for grid points from TIN data structure
- Methods of data pre-processing using TIN, with the aim of forming an additional set of data (structural terrain lines and points in parts with rare starting data) and their use for interpolation of 3D models using some of the previous procedures

Choosing the appropriate interpolation method is not easy and requires a good knowledge of the characteristics of the terrain being modelled, the characteristics of the input data as well as the characteristics of the available interpolation methods. For interpolation of heights in points of 3D models, all methods can be used which, on the basis of heights given in arbitrarily distributed points, the height for any set point that falls within the area are covered by the input data. Some of these methods have been specially developed for interpolation of heights at grid points.

6.6.2.1 Basic Principles of Variogram Calculation

The introduction showed us how geostatisticians observe the spatial dependence of sampled or analytical values. The variogram is the basic means of evaluation, quantification and spatial dependence. In practice, the variogram represents the root mean square of the difference of two values calculated as a function of the distance of these values. It is the basis of all geostatistical calculations (Wellmer 1998).

The set of all data pairs at the same distance is called the class, and by merging the values for each class, the curve of the experimental variogram is obtained. There are four parameters that can be read from the variogram, and they are (1) deviation ("Nugget"), (2) threshold ("Sill"), (3) range ("Range") and (4) distance ("Distance"). (1) Deviation ("Nugget") is a positive value on the y-axis, where the variogram curve intersects that axis. (2) The threshold ("Sill") is the value corresponding to the variance. Once the curve reaches the threshold, it stops growing properly and generally begins to oscillate around the threshold. (3) Range is the distance along the x-axis from zero to the point where the variogram curve intersects the threshold.

Reach represents the continuity between points of adjacent data. At a greater distance from the intersection of the threshold and the variogram curve, one can no longer speak of continuity between points. (4) "Distance" is the distance on the x-axis at which the data in the variogram direction are compared. Each distance makes one class. This value is often assigned a certain tolerance (offset) in order to increase the number of input data. Thus, we add a certain value of the offset to the class boundary, so the variogram classes are represented by certain intervals (e.g. 0.5–1.5, 1.5–2.5, etc.). Usually the offset is set to 1/2 distance values, which maximizes the number of pairs and at the same time the reliability of the spatial analysis (Wellmer 1998).

6.6.2.2 Kriging

Namely, the kriging method is based on the use of known values of so-called variable control points, the influence of which on the assessment is expressed by appropriate weighting coefficients (Wellmer 1998; de Smith et al. 2009). The most demanding procedure in kriging is to determine the weighting coefficients for each control point individually. During the assessment, it is necessary to meet certain criteria, i.e. to be impartial and defined so that the variance of the difference between the actual and estimated values at the selected points is the smallest (Zeigler et al. 2000). Due to reliable estimates of spatially distributed variables, the kriging method has found great application in many branches of research. It was primarily created for the needs of mobile environments and surfaces in mining and geology, for example, as a means of improving the assessment of ore reserves and natural resources. The basic equation of the kriging method is given by a mathematical expression:

$$z\left(s_0\right)=m\left(s_0\right)+x\left(s_0\right)+e\left(s_0\right)$$

where:

$z(s_0)$ – a statistical representation of a surface that with a high degree of probability approximates the actual surface for which data were collected

$m(s_0)$ – surface trend, i.e. quantification of the spatial structure of the surface with (co)

$x(s_0)$ – stochastic part, i.e. evaluation of the value of the function surfaces at given points

$e(s_0)$ – noise, i.e. interference with perception

The most significant property of this interpolation method is to keep the measured quantities as fixed, which means that it includes the original data set that is in the interpolation process will not change. Relationships between existing and estimated values are expressed values of covariance or variogram. Spacious dependence is usually expressed mathematically in form certain functions of spatial coherence such as a semi-variogram or covariance function. Semi-variogram and covariance function are suitable tools in data analysis and clarification. Even more, the coefficients are weights assigned to interpolation points in kriging. This determined the influence of the known value on the estimated value with respect to their distance (Zeigler et al. 2000).

Also, several techniques or variants of the kriging method have been developed in order to adapt the initial algorithm to the requirements of different data. The most famous are *simple kriging*, *ordinary kriging* and *universal kriging*. Simple kriging is based on the assumption that the covariogram is a decreasing function of distance. This is the result of the assumption about the stationarity of the observed function. The trend is represented by a constant value that is known in advance. The second variant of the kriging method, i.e. ordinary kriging, is especially interesting. Namely, in ordinary kriging, we start from the assumption of quasi-stationarity. It requires that the increments of the function be stationary, but not the function itself. The trend is constant and not known, so it should be assessed on the basis of data. Universal kriging is less restrictive than simple and ordinary kriging, and it assumes only the stationary increment of the regionalized variable and only in the neighbourhood of the observed point (Zeigler et al. 2000; Li et al. 2005).

6.7 Hunting the Anthropogenic Depositions in Mine Environs

Atmospheric deposition poses significant ecological concerns. In this work, attic dust was examined as historical archive of anthropogenic emissions, with the aim of elucidating the pathways of enrichments associated with exploitation of Cu, Pb and Zn minerals in the Bregalnica river basin region. Attic dust samples were collected from 84 settlements. At each location for attic dust sampling, topsoil samples from the house yards were also collected. Mass spectrometry with inductively coupled plasma (ICP-MS) was applied as an analytical technique for the determination of Ag, Bi, Cd, Cu, In, Mn, Pb, Sb, Te, W and Zn. The universal kriging method with linear variogram interpolation was applied for the construction of spatial distribution maps.

6.7.1 Characterization of Investigated Area

The investigated area includes the basin of the river Bregalnica which is found in the area of the east planning region of the Republic of Macedonia (N: 41°27′-42°09′ and E: 22°55′-23°01′), covering ~4000 km^2 (Fig. 6.2). The region of the investigated area is geographically composed of several sub-regions. The area is characterized by two valleys – Maleševska and Kočani valleys. The Maleševska valley represents the upper course of the river Bregalnica where the river source is also

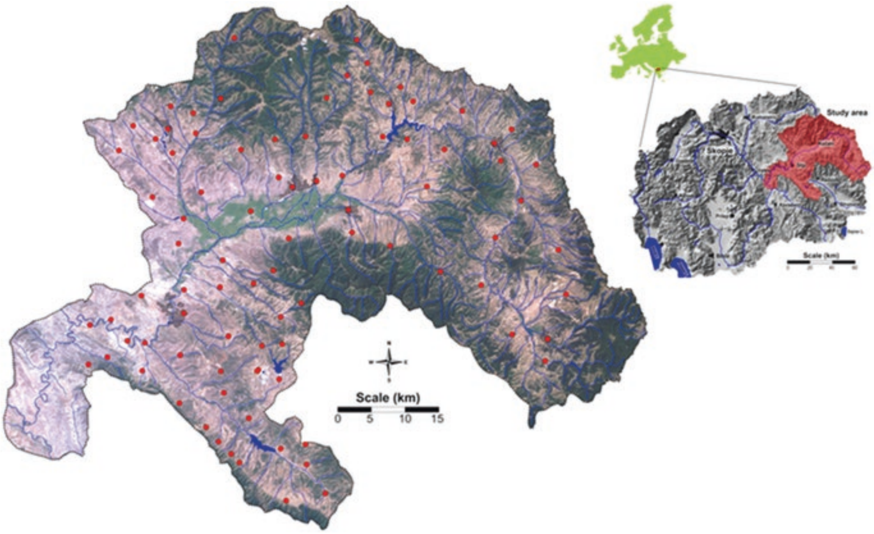

Fig. 6.2 Location of the investigating area and sampling network of attic dust and soil samples

located, with altitude range of 700–1140 m. The valley is enclosed by the Maleševski Mountains on the east, by the Ograzhden Mountain on the south-southeast and by the Plačkovica and Obozna on the west. The average annual precipitation amounts to about 500 mm with significant variations from year to year, as well as in the different sub-regions (Lazarevski 1993). The precipitation is mostly related to and conditioned by the Mediterranean cyclones (Lazarevski 1993). During the summer period, the region is most often found in the centre of the subtropical anticyclone, which causes warm and dry summers. From the central area of the region, as the driest area, the average annual precipitation increases in all directions, because of either the increase in the influence of the Mediterranean climate or the increase in altitude (Lazarevski 1993). In the region are distinguished about ten climatic-vegetation soil areas with considerably heterogeneous climate, soil and vegetation characteristics (Lazarevski 1993). Regarding the demographic structure in the region of the Bregalnica basin, only 4% of the populated areas belong to the category of urban areas, while 96% of the total populated areas are categorized as rural areas. Considering land use, the region is considerably diverse. Along the course of the Bregalnica river dominate agricultural cultivated lands. About 30% belong to the forest regions, localized around the Maleševska valley, represented by the Maleševski Mountains, and then around the Kočani valley, represented on the one side by the Osogovo Mountains and by Plačkovica on the other side. The investigated area that covers the basin of the river Bregalnica lies on the two main tectonic units – the Serbian-Macedonian massive and the Vardar zone (Dumurdzanov et al. 2004). The polyphasal Neogene deformations through the insignificant movements associated with the volcanic activities had direct influence on the gradual formation of the reefs and the formation of deposits in the existing basins. From the middle

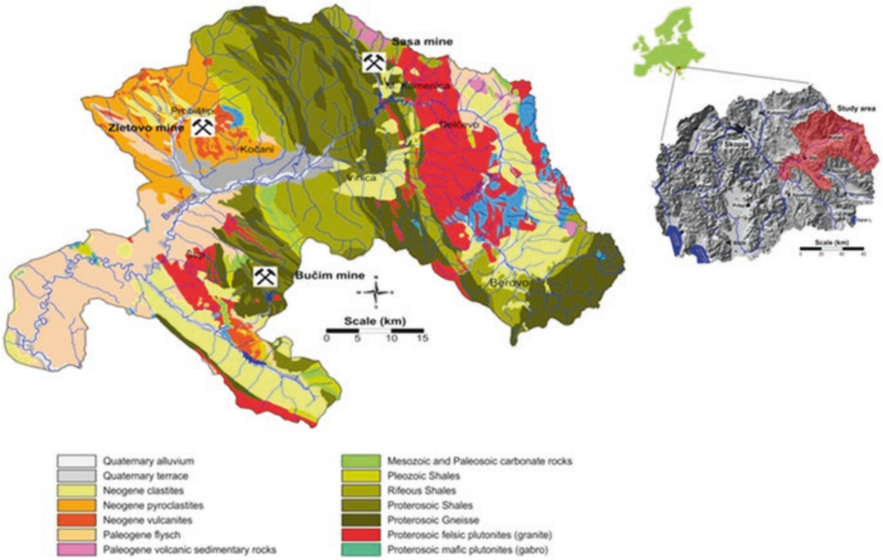

Fig. 6.3 Generalized geology of the investigated area and location of the emission sources – *Sasa* mine (Pb-Zn hydrothermal exploitation), Kratovo-Zletovo district and Bučim mine area (Cu-Au hydrothermal exploitation)

Miocene to the end of the Pleistocene, there were alternating periods of fast and slow landslide accompanied with variable sedimentation (deposition). The Cenozoic volcanism represents a more recent extension in the Serbian-Macedonian massive and the Vardar zone. The oldest volcanic rocks occur in the areas of Bučim, Damjan, the Borov Dol district and in the zone of Toranica, Sasa, Delčevo and Pehčevo (Dumurdzanov et al. 2004). These older volcanic rocks were formed in the middle Miocene from sedimentary rocks that represent the upper age limit of the rocks. The origin of these oldest volcanic rocks is related to the Oligocene – the early Miocene period. Volcanic rocks are categorized as follows: andesite, latite, quartz latite and dacite. Volcanism appears sequentially and, in several phases, forming sub-volcanic areas. On the other hand, the pyroclastites are most frequently found in the Kratovo-Zletovo volcanic area, where the dacites and andesites are the oldest formations (Fig. 6.3).

6.7.2 Sampling Protocol and Analytics

Attic dust samples were collected from the attics from total of 84 houses built between 1920 and 1970 (Fig. 6.2). The data information for house building period were obtained from the local population at the time of the sampling. The collection of attic dust samples was performed according to the adopted protocol given by Šajn

(2003). The sampling protocol includes (a) removing the dinginess from the attic surface, (b) collecting the corpuscular dust form the attic surface using plastic brush and (c) transferring the collected dust in polyethylene bags. The representative sample from one house was obtained collecting the dust from five to ten attics, depending of the house condition.

The soil samples brought in the laboratory were subjected to cleaning and homogenization, drying at room temperature, or in a drying room at 40 °C, to a constantly dry mass. Then the samples were passed through a 2 mm sieve and finally were homogenized by grinding in a porcelain mortar until reaching a final size of the particles of 125 μm. Following the physical preparation, the samples were chemically prepared by wet digestion, applying a mixture of acids in accordance with the international standards (ISO 14869-1: 2001).

For digestion of attic dust and soil samples, open wet digestion with mixture of acids was applied. Precisely measured mass of dust samples (0.5 g) was placed in Teflon vessels, and 5 mL concentrated nitric acid (HNO_3) was added, until the brown vapours came out from the vessels. Nitric acid is very suitable oxidant for digestion of environmental samples. For total digestion of inorganic components, 5–10 mL hydrofluoric acid was added. When the digest became clear solution, 2 mL of $HClO_4$ was added. Perchloric acid was used for total digestion of organic matter. After 15 minutes cooling the vessels, 2 mL of HCl and 5 mL of H_2O were added for total dissolve of metal ions. Finally, the vessels were cooled and digests quantitatively transferred to 50 mL calibrated flasks. In this way, the digested soil and sediment samples were prepared for determining the contents of the different elements using atomic emission and mass spectrometry.

PerkinElmer SCIEX ELAN DRC II (Canada) inductively coupled plasma mass spectrometer (ICP-MS, with quadrupole as single detector) was used for measurement of the concentration trace elements, while for the major elements' content determination, atomic emission with inductively coupled plasma (ICP-AES, Varian 715ES) was applied. For this study, reagents with analytical grade, namely, nitric acid, trace pure (Merck, Germany); hydrofluoric acid, p.a. (Merck, Germany); perchloric acid, p.a. (Merck, Germany); hydrochloric acid, p.a. (Merck, Germany); and redistilled water, were used for the preparation of all solutions. Standard solutions of metals were prepared by dilution of 1000 mg/L solutions (11355-ICP multielement standard). For each element analysed, previous optimization of the instrumental conditions was performed.

6.7.3 Data Processing Methods

After the instrumental analysis, the values obtained contain the contents of the elements, initially co-transformed and normalized. The processing of numerous variables with a heterogeneous distribution structure is often complex and requires careful processing. On the other hand, there is significant deviations from the normal distribution of data. This state of the obtained variables was improved by applying the transformation of the data set. For the normalization of a heterogeneous set of values, the statistical method of Box-Cox is used (Box and Cox 1964). Normalized values are processed with basic descriptive statistics, using the basic parameters of this method.

Bivariate analysis is used to perform a comparative analysis of the dependencies between variables (set of values for one variable). Two-dimensional scatter-plots are used to display the correlations and identify them visually.

Multivariate analysis is applied to extract dominant associations of variables (Šajn 2006). As a measure of similarity between variables, the product-moment correlation coefficient (*r*) was applied. There are various rotational strategies that have been proposed (Šajn 2006; Žibret and Šajn 2010). The purpose of the applied statistical data processing models is to obtain the most realistic model of multi-dimensional variable distribution system. For variables, i.e. the data set for the content of a given element, for which the applied factor analysis will deposit low values, they will be excluded from further analysis. Factor analysis used orthogonal varimax rotation. Factor analysis is an interdependence technique because it looks for a group of variables that are similar in that they "move together" and therefore have great interdependence. When one variable has a large value, then the other variables in the group have a large value. For the effective application of factor analysis, as well as other multivariate interdependence techniques, it is necessary to have a minimal amount of redundancy of variables, that is, the variables at least slightly overlap in their meaning. Thanks to this redundancy, it is possible to discover a pattern in the behaviour of variables, that is, the basic idea (factor) by which they are imbued (Žibret and Šajn 2010). Cluster analysis is a statistical technique used to identify how different entities – variables – can be grouped together for the sake of their characteristics. Also known as clustering, it is a preliminary data analysis tool that aims to sort different objects in a group in such a way that those who belong to the same group have the highest degree of association. The most commonly processed dendrograms or clusters are expressed by distance, Dlink/Dmax (%).

6.7.4 Evaluation of Data Summary Matrix

Data summary was introduced by Balabanova et al. (2019) generating the following geochemical associations: F1, Ga-Nb-Ta-Y-(La-Gd)-(Eu-Lu); F2, Be-Cr-Li-Mg-Ni; F3, Ag-Bi-Cd-Cu-In-Mn-Pb-Sb-Te-W-Zn; F4, Ba-Cs-Hf-Pd-Rb-Sr-Tl-Zr; F5, As-Co-Ge-V; and F6, K-Na-Sc-Ti. The total variability for dominant loadings of 81.5% was established (Balabanova et al. 2019).

6.7.4.1 Tracking the Lithogenic Anomalies in the Investigated Area

Multivariate analysis successfully reduced variables by extracting dominant associations of elements. Detailed analysis of these associations, through thorough study and comparative analysis with typical lithology and geologically dominant formations, enabled the generation of the following analytical qualifications. Factor 1 [Ga-Nb-Ta-Y-(La-Gd)-(Eu-Lu)] clearly expresses the typical connection of some basic earth forming process, due to the incorporation of the rare earth. Their sources are mainly natural phenomena such as rock weathering and chemical processes in soil. The second geochemical association (Be-Cr-Li-Mg-Ni) relays also on the effect from the windblown dust from the surface soil layers. These determined elements are considered "natural" because their origin is primarily crustal-soil particles suspended and transported by wind. High factor loadings are related to some old formation such as Paleogene flysch and Quaternary unconsolidated sediments. The occurrence of Factor 4 (Ba-Cs-Sr-Rb-Pd-Tl-Zr-Hf) presents an interesting geochemical association, incorporating the incompatible/hygromagmatophile elements. Element enrichments occur in areas with predominance of Neogene pyroclastite and sediments. According to generalized geology map (Fig. 6.3), Kratovo-Zletovo region is the unique district in the region located along the continental margin and is closely related to the Tertiary volcanoes and hydrothermal activities in this area. The sixth factor includes K-Na-Sc-Ti. Although it is the least distinguished factor; nevertheless, this geochemical association is related with the lithogenic dominance of the Neogene sediments and Paleogene flysch. Their occurrence in the environment is usually correlated with the major minerals in basaltic and ultramafic rocks which have two kinds of cationic lattice sites: small tetrahedral sites occupied by Si and Al (Balabanova et al. 2019).

6.7.4.2 Selecting Dominant and Silent Anthropogenic Markers

One of the more important goals of this research is to determine the success of this multidisciplinary approach in determining anthropogenic markers (dominant and recessive). Dominant and recessive markers of anthropogenic inputs were identified after multivariate extraction. Factor 3 (Ag-Bi-Cd-Cu-In-Mn-Pb-Sb-Te-W-Zn) was the most heterogenic geochemical association (Table 6.1). Enriched deposition occurred dominantly on Rifeous shale. These very old rocks occur as transition lithologic units between the Neogene and Paleogene volcanism in the investigated area. Pb-Zn mines *Sasa* and *Zletovo* are located in the areas with dominantly presence of the Oligocene and Neogene volcanism appears sequentially and, in several cases in sub-volcanic areas. On the other hand, the pyroclastites are most frequently found in the Kratovo-Zletovo volcanic area, where the dacites and andesites are the oldest formations. This geochemical association links typical elements which are normally associated with air pollution (Cd-Pb-Zn) and usually are not influenced by lithological background. Spatial patterns show intensive deposition in the area of poly-metallic hydrothermal exploitations, *Sasa*, *Zletovo* and *Bučim*. This geochemi-

Table 6.1 Element distribution according to dominant geological formation in the area of the elements from Factor 3 (median values are given; $N = 84$)

	Unit	River terraces (Q)	River sediment (Ng)	Pyroclastite (Ng)	Flysch (Pg)	Schists (Pz)	Schists (R)	Schists (Pt)	Gneisses (Pt)	Granite (Pt)
Ag	mg/kg	0.53	0.48	0.59	0.45	0.60	1.3	0.44	0.43	0.58
Bi	mg/kg	0.28	0.28	0.31	0.29	0.26	0.61	0.45	0.35	0.22
Cd	mg/kg	0.42	0.45	0.44	0.52	0.33	1.5	0.28	0.25	0.31
Cu	mg/kg	40	25	27	24	25	43	49	26	29
In	µg/kg	35	28	46	37	42	94	46	34	39
Mn	mg/kg	440	450	540	480	560	830	530	370	430
Pb	mg/kg	54	83	48	18	30	110	55	22	12
Sb	mg/kg	0.80	0.64	0.89	0.62	0.55	1.1	0.66	0.45	0.46
Te	µg/kg	34	15	30	27	28	63	41	21	19
W	mg/kg	0.85	0.79	1.0	0.76	1.0	2.0	1.1	1.1	0.76
Zn	mg/kg	110	130	98	110	90	220	86	72	88

Q Quaternary, *Ng* Neogene, *Pg* Paleogene, *R* Rifeous, *Pt* Proterozoic, *Pz* Paleozoic cal occurrence can be used as a poly-metallic marker for anthropogenic emissions. This geochemical association unite some “not very common” elements for air pollution (argentum, indium and tellurium). Generally, most of the trace element distribution is characterized mainly in terms of the different lithological units, as in the case of In (42 μg/kg), Bi (0.30 mg/kg) and Ag (0.54 mg/kg) distribution differs from the rest of the trace elements, due to the dominance of the atmospheric deposition (with median of 31 μg/kg and max. Value of 1300 μg/kg) vs. soil distribution (median of 17 μg/kg and max value of 280 μg/kg). Copper contents in air-distributed dust range from 6.7 to 880 mg/kg, while in the topsoil layer, an extended distribution has occurred to 1200 mg/kg. Enriched values for the Cu content in attic dust were obtained from houses located very close to Cu mine *Bučim*. Cadmium contents also have an enrichment trend in air-distributed dust (0.054–25 mg/kg); but cadmium content in the topsoil layer does not indicate any significant enrichment (0.005–9 mg/kg). The distribution of lead and zinc is similar to that of copper and cadmium in the investigated area. Lead content ranges from 0.005 to 3900 mg/kg, while Zn contents show lower enrichments from 26 to 3200 mg/kg. Lithogenic distribution in soil reaches 1200 mg/kg and 590 mg/kg, respectively, for Pb and Zn. According to the dominant lithological units, Rifeous shales are mostly enriched with Pb and Zn, where the median values for Pb and Zn contents are 110–220 mg/kg, respectively. For the spatial distribution of the factor scores, universal kriging with the linear variogram interpolation method was applied for the construction of maps showing the spatial distribution of elemental distribution. The significance is given on those areas where the content of the elements exceeds the 75th percentile of the data distribution for the element content. Attic dust’s element distribution is marked with number 1 at each figure for element distribution. In order to distinguish the lithogenic impact on the element distribution, the topsoil layer was also investigated for the content of the elements (marked with number 2 at each figure for element distribution). Also, this investigation fortifies an extended anthropogenic association (Ag, Bi, In and Mn) that implement some other anthropogenic activities such as agricultural activities (use of urban sludge, manure and phosphate fertilizers) and their occurrence can be a secondary affection from mine poly-metallic pollution (Balabanova et al. 2019). Affiliation of tellurium and wolfram to this group spread up an elemental anomaly in the Berovo region. For all elements that associate in this group, enriched value deposition occurs in this mentioned area. Almost 20 years ago, Arsovski (1997) points to a poly-metallic enrichment in this area, so-called Vladimirovo-Berovo, during the tectonic investigation. The present investigation also interpolates this area as a metallic−/metalloid-enriched zone, with emphasis on the anthropogenic elements. This occurrence was also determined for the geochemical marker (As-Co-Ge-V) established by Balabanova et al. (2019). Spatial mappings for each element are presented in Figs. 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 6.10, 6.11, 6.12, 6.13, and 6.14.

Geochemical association given as Factor 5 (Balabanova et al. 2019) associates the following elements, As, Co, Ge and V, with predominant occurrence on Rifeous shales (Table 6.2). This kind of geochemical *fingerprinting* occurs along the whole course of the Bregalnica river. Accordingly, the resulting areal distribution map used

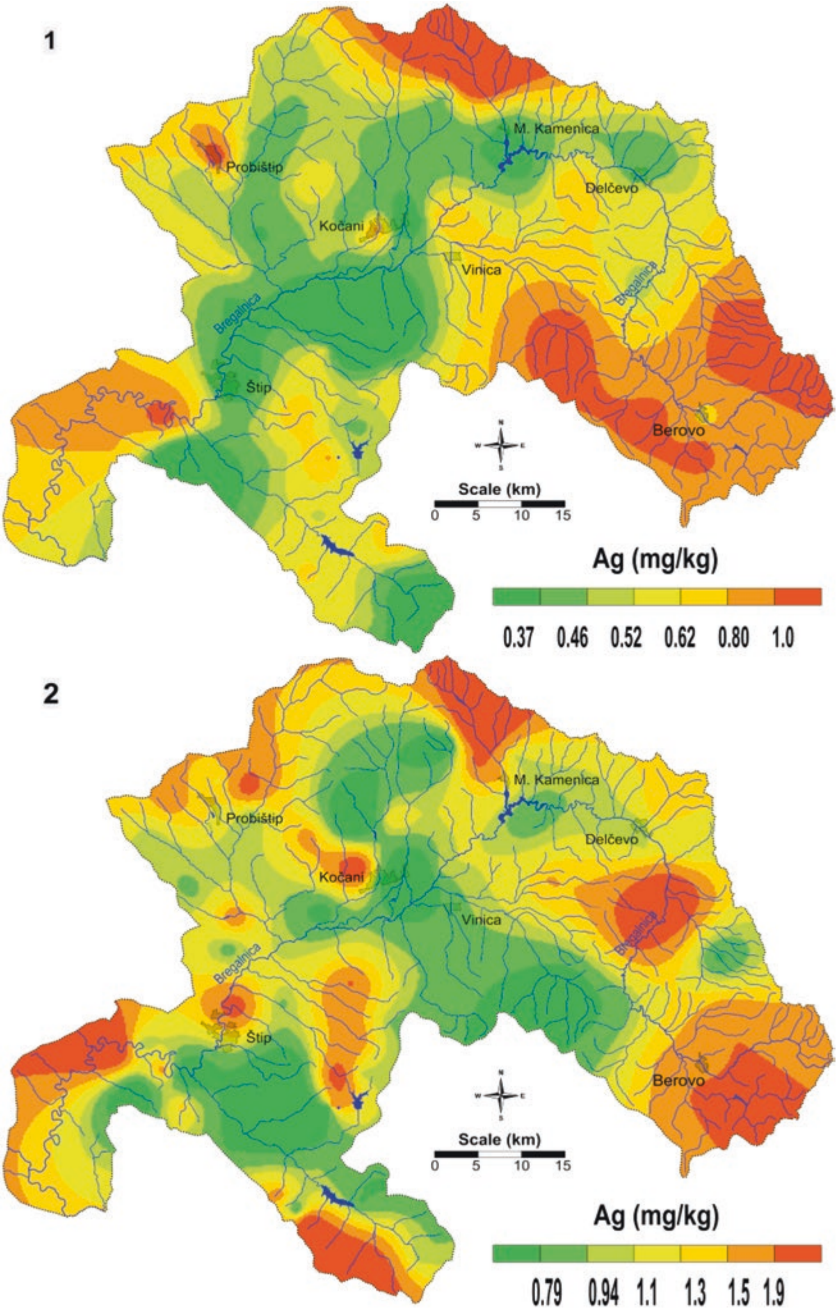

Fig. 6.4 Spatial mapping with *kriging* method, for distribution of silver in attic dust (1) and soil (2)

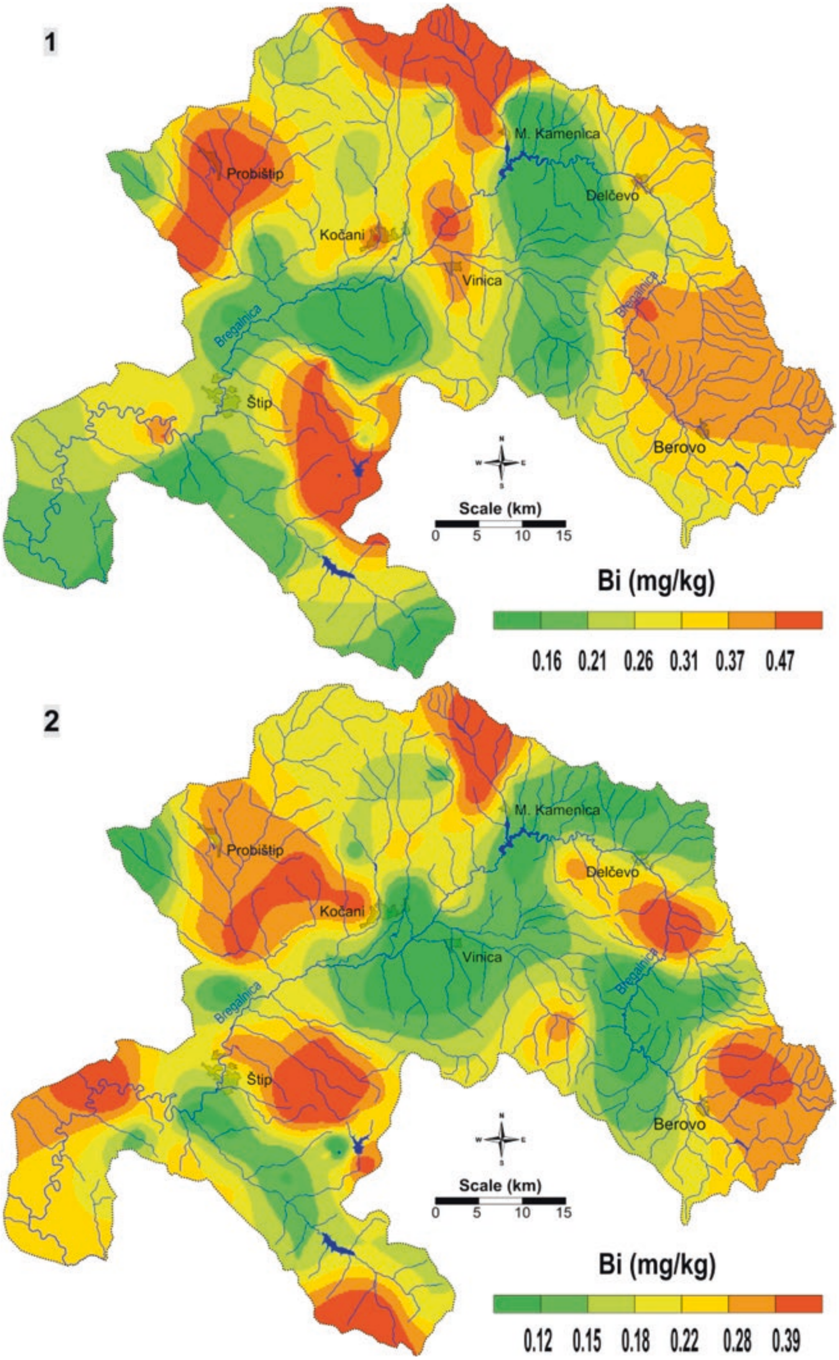

Fig. 6.5 Spatial mapping with *kriging* method, for distribution of bismuth in attic dust (1) and soil (2)

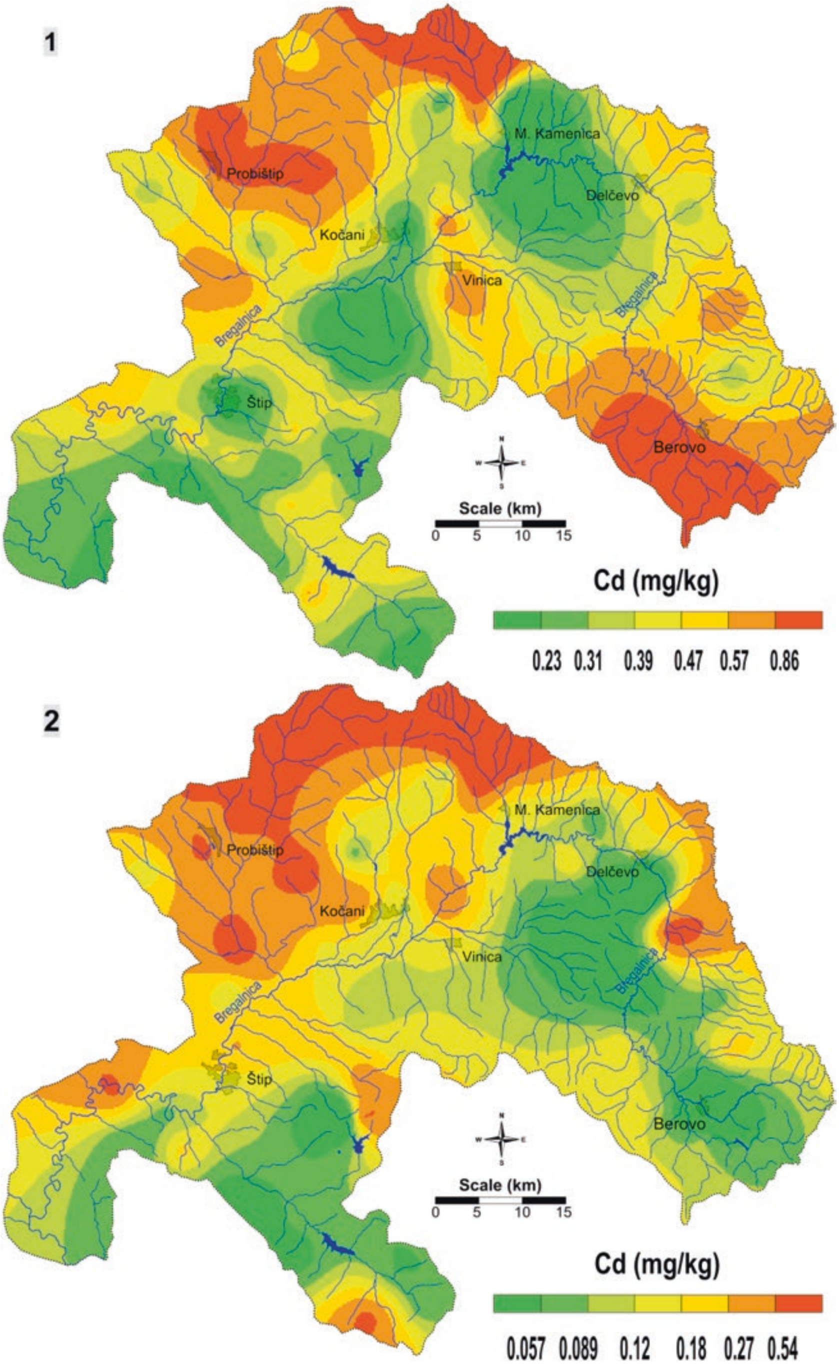

Fig. 6.6 Spatial mapping with *kriging* method, for distribution of cadmium in attic dust (1) and soil (2)

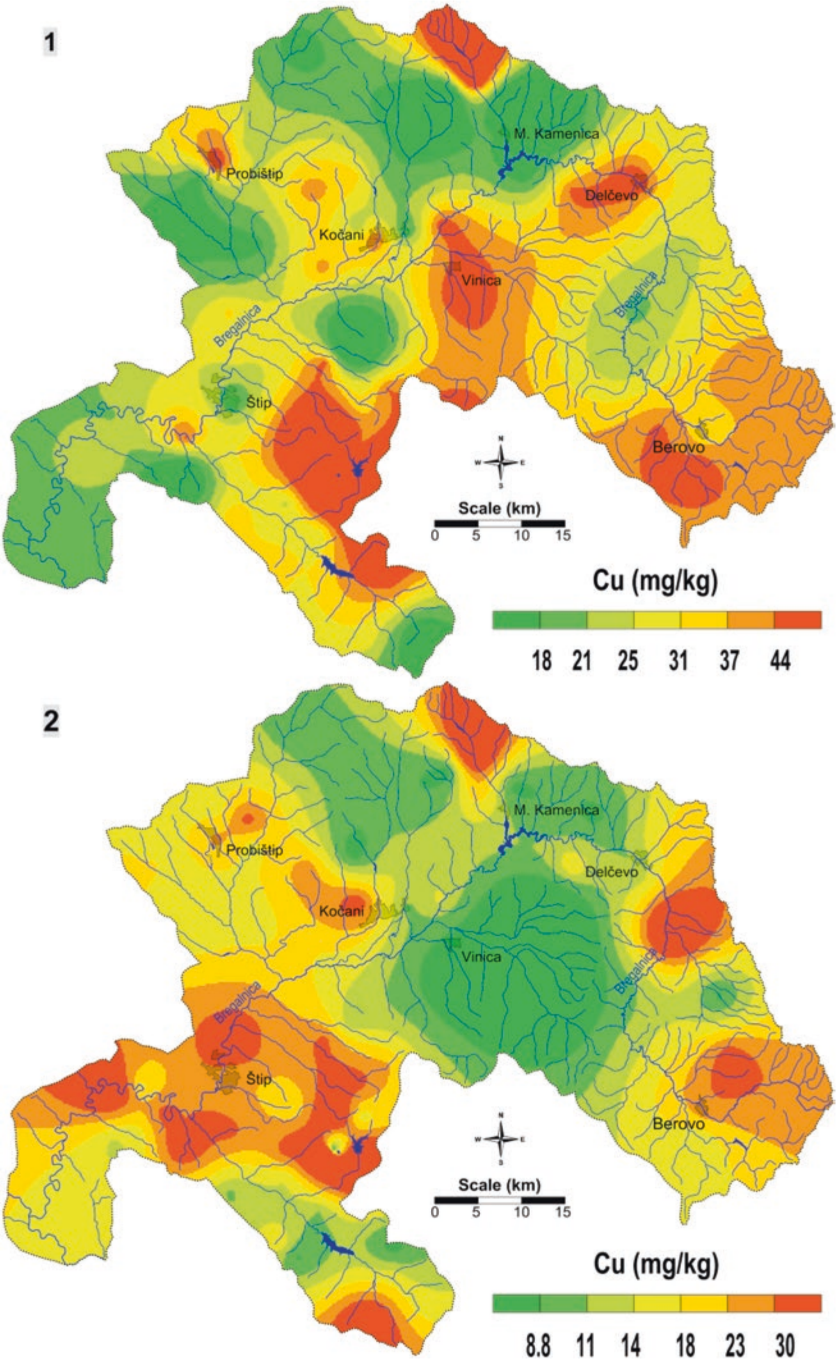

Fig. 6.7 Spatial mapping with *kriging* method, for distribution of copper in attic dust (1) and soil (2)

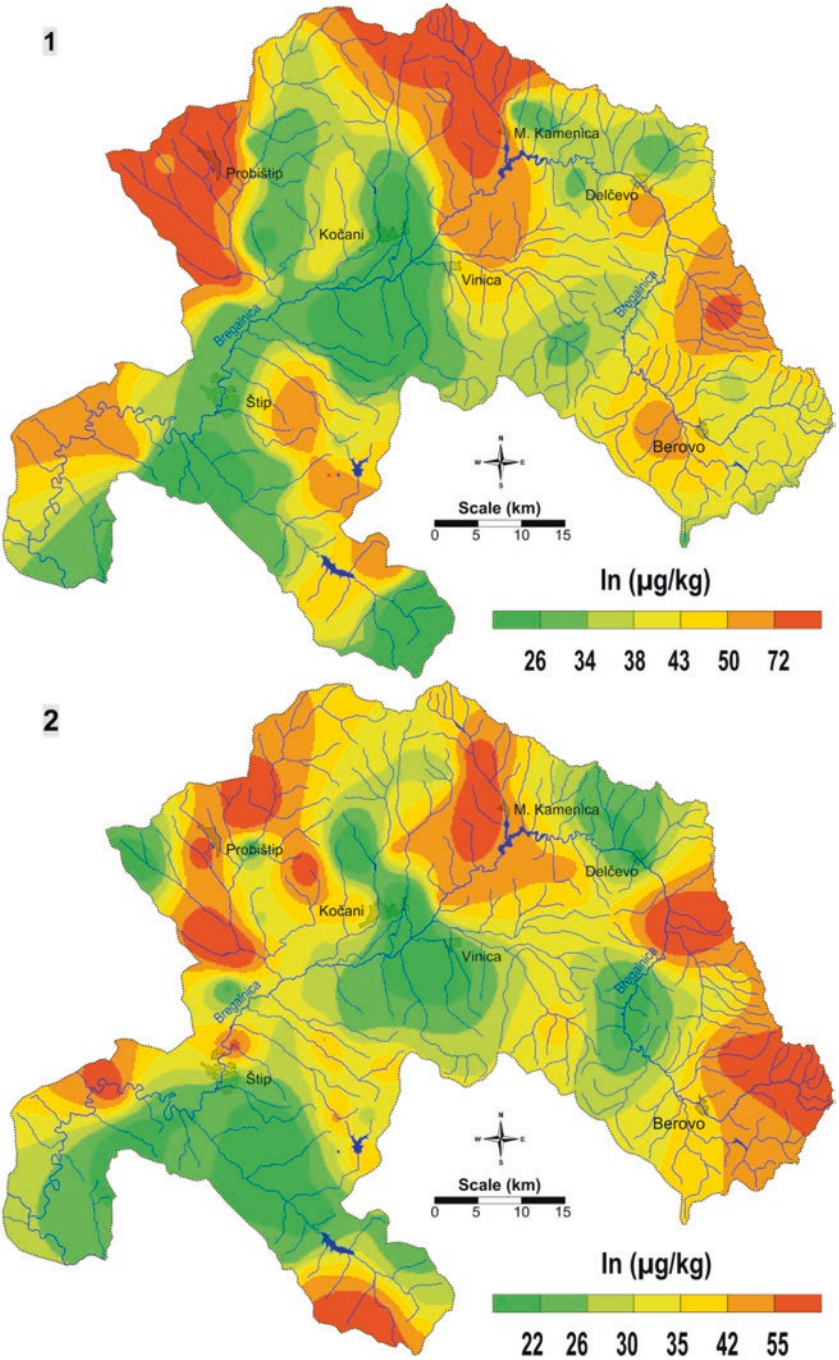

Fig. 6.8 Spatial mapping with *kriging* method, for distribution of indium in attic dust (1) and soil (2)

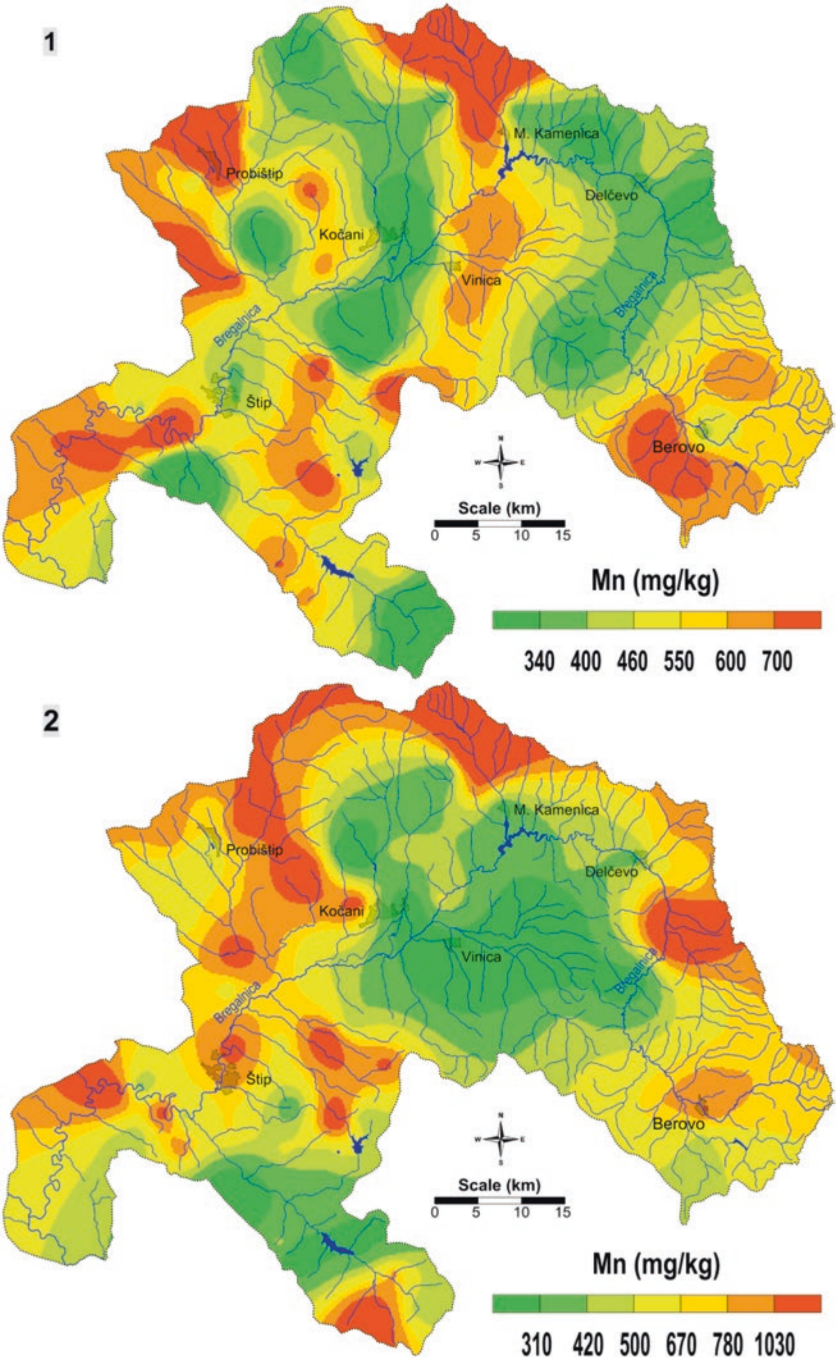

Fig. 6.9 Spatial mapping with *kriging* method, for distribution of manganese in attic dust (1) and soil (2)

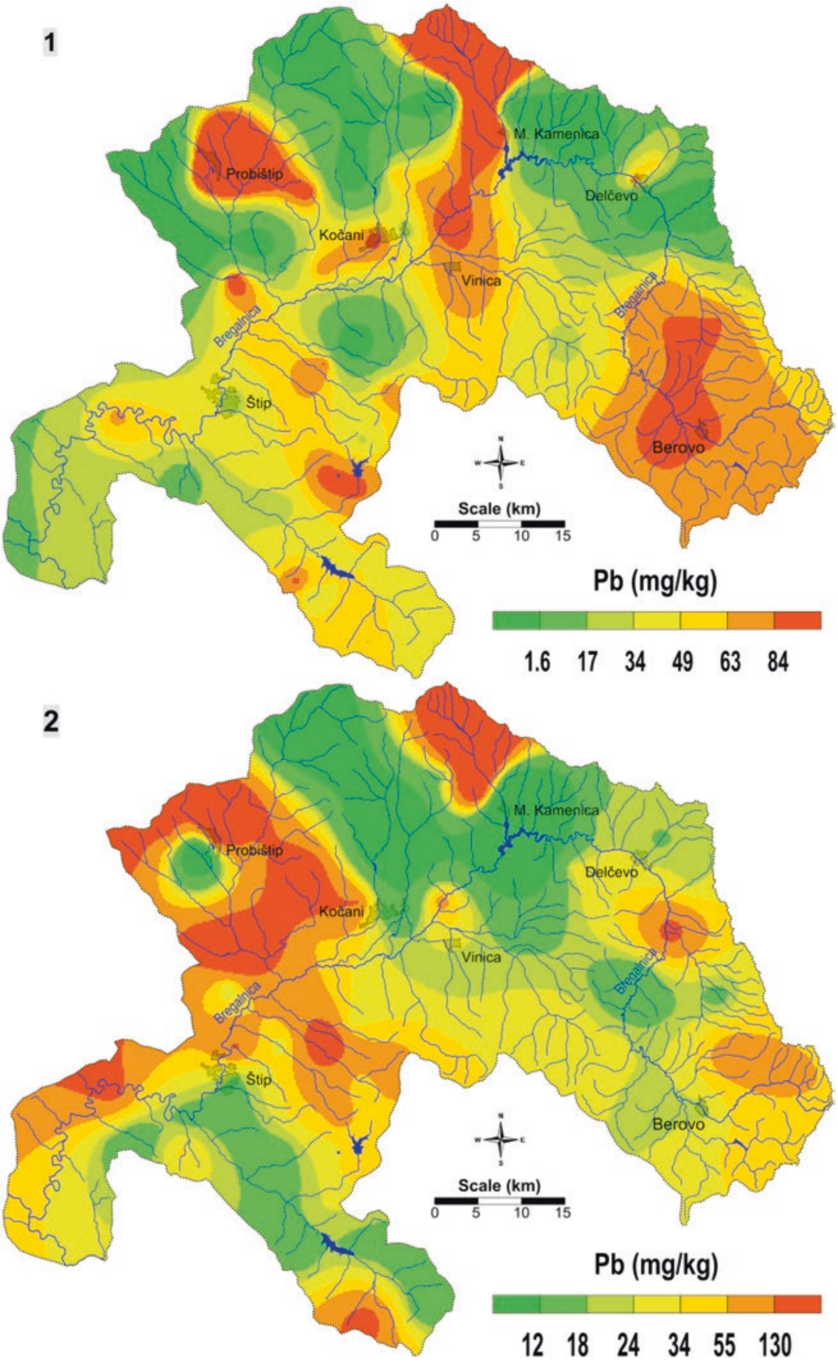

Fig. 6.10 Spatial mapping with *kriging* method, for distribution of lead in attic dust (1) and soil (2)

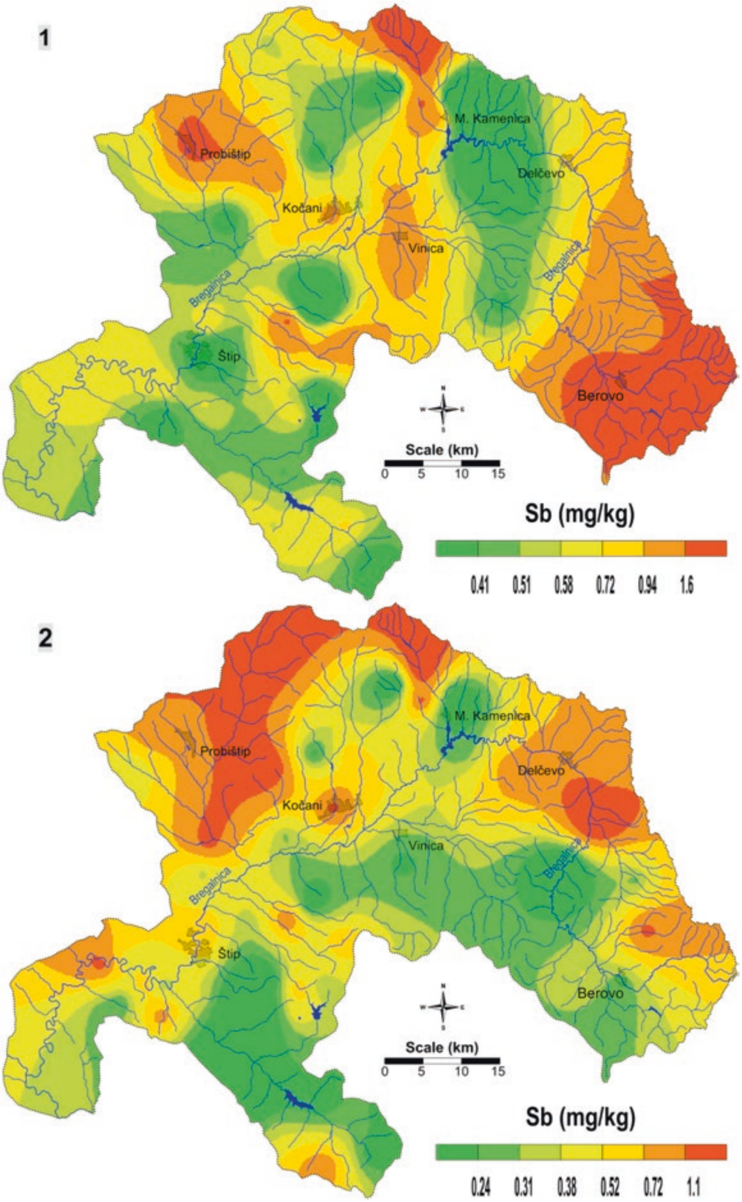

Fig. 6.11 Spatial mapping with *kriging* method, for distribution of antimony in attic dust (1) and soil (2)

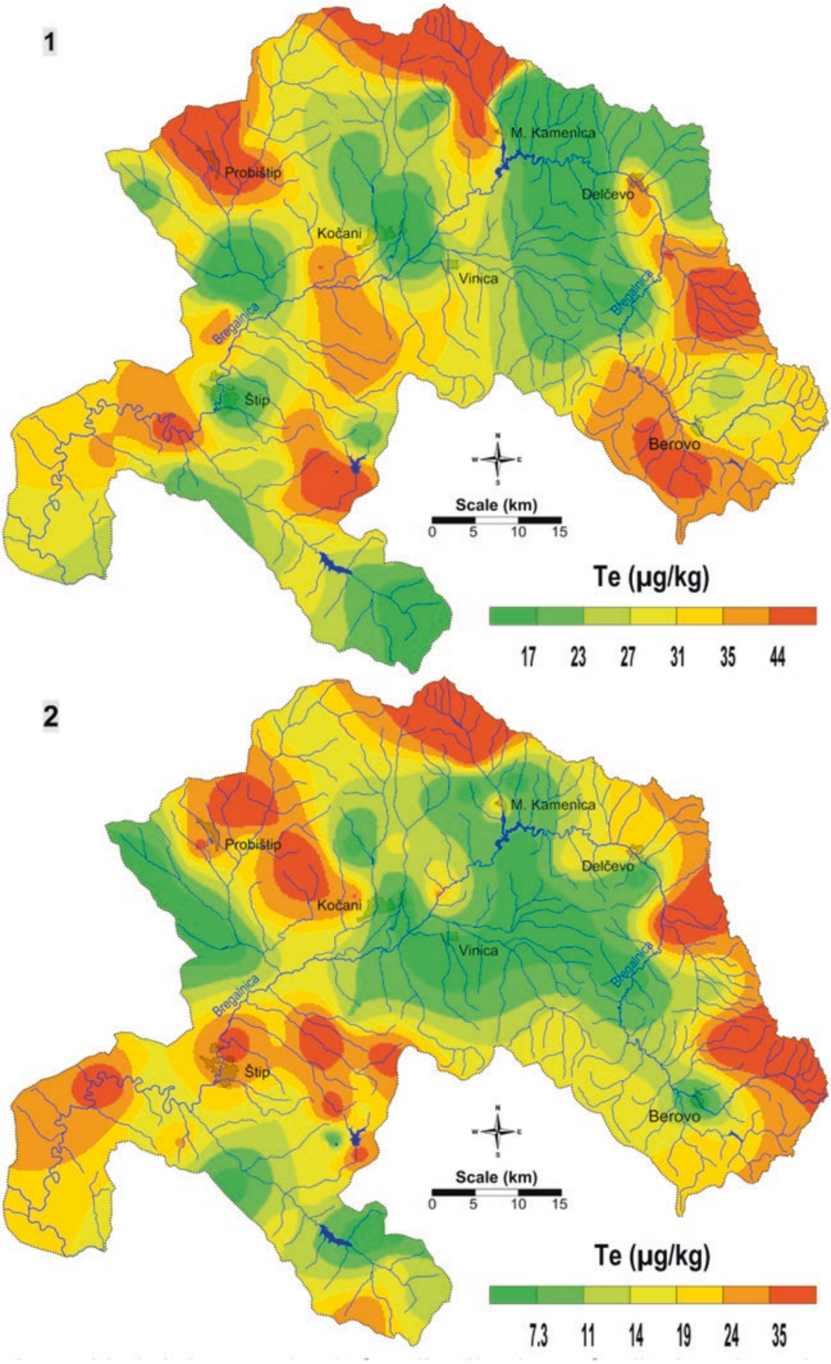

Fig. 6.12 Spatial mapping with *kriging* method, for distribution of tellurium in attic dust (1) and soil (2)

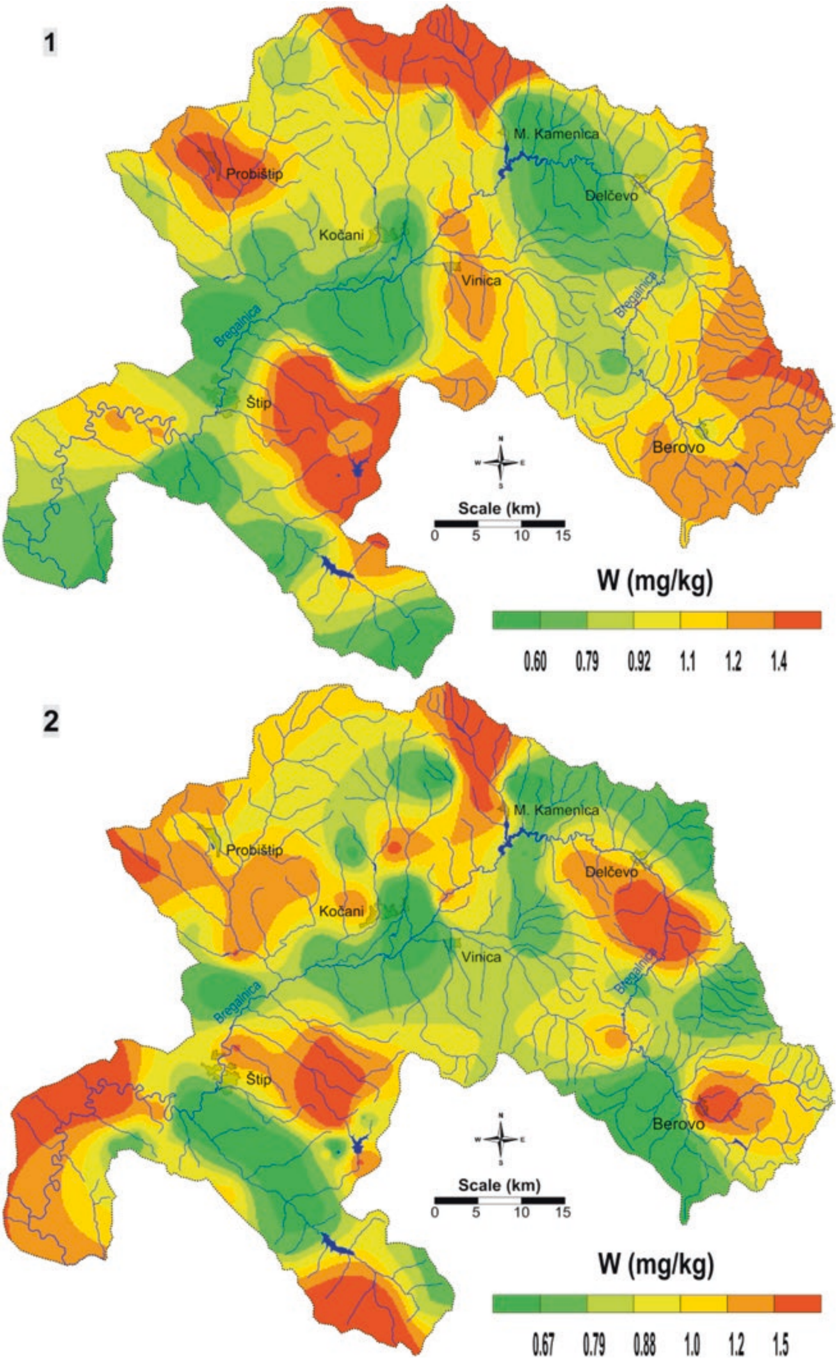

Fig. 6.13 Spatial mapping with *kriging* method, for distribution of wolfram in attic dust (1) and soil (2)

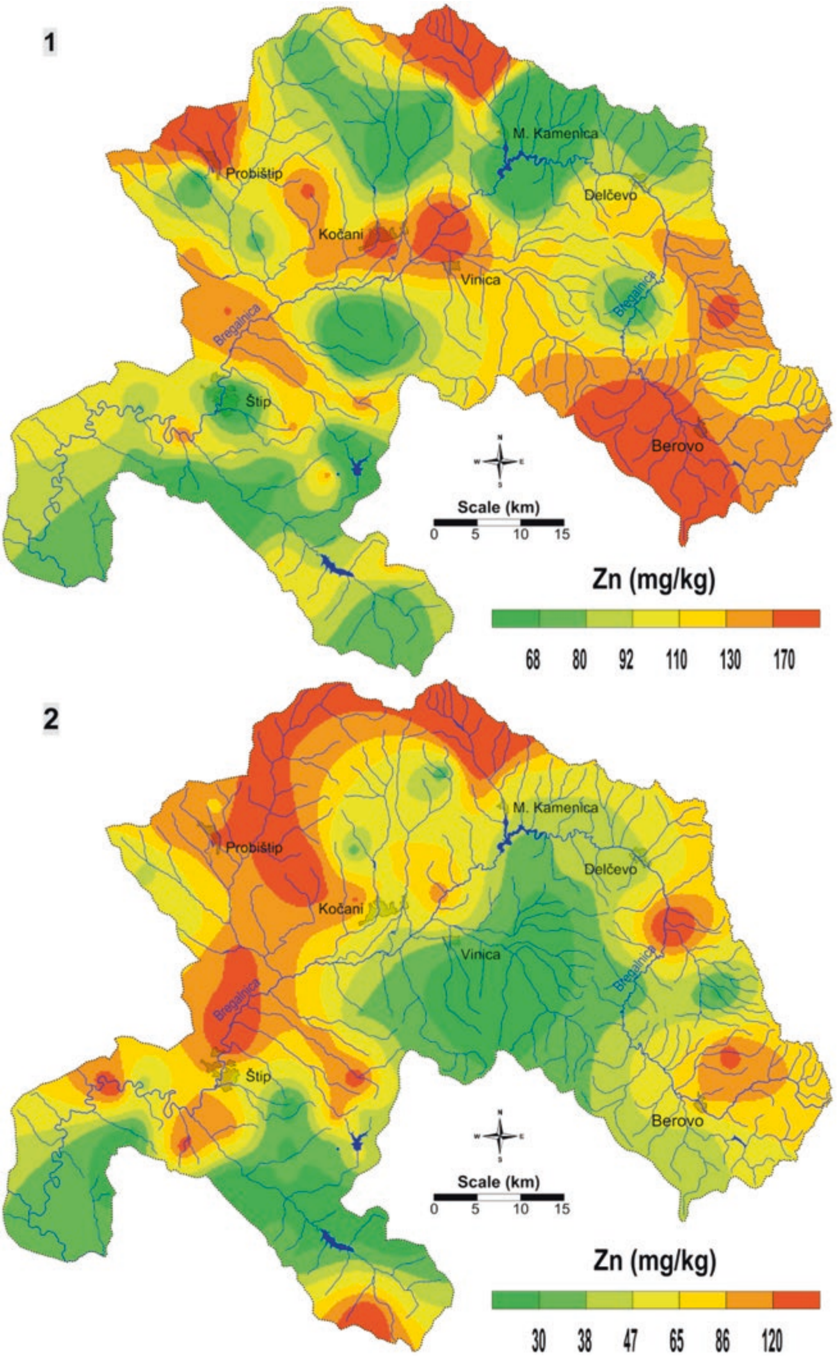

Fig. 6.14 Spatial mapping with *kriging* method, for distribution of zinc in attic dust (1) and soil (2)

Table 6.2 Element distribution according to dominant geological formation in the area of the elements from Factor 5 (median values are given)

	Unit	River terraces (Q)	River sediment (Ng)	Pyroclastite (Ng)	Flysch (Pg)	Schists (Pz)	Schists (R)	Schists (Pt)	Gneisses (Pt)	Granite (Pt)
As	mg/kg	19	23	16	21	16	26	20	15	14
Co	mg/kg	6.6	5.6	7.2	6.0	7.3	9.1	6.4	6.5	7.6
Ge	mg/kg	0.53	0.51	0.60	0.55	0.65	0.75	0.64	0.52	0.52
V	mg/kg	68	100	79	68	74	75	87	76	71

Q Quaternary, *Ng* Neogene, *Pg* Paleogene, *R* Rifeous, *Pt* Proterozoic, *Pz* Paleozoic to support with high certainty the assessment for poly-metallic enrichments as ascribed to urbanization, including vehicular emissions and incinerators and industry. Comparative analysis for the areal patterns singled out a very similar behaviour of the Factor 3 and Factor 5. Clustering method was very useful for the determination of both geochemical associations. Furthermore, there is a strong interconnection between the anthropogenic and lithogenic fingerprinting (Balabanova et al. 2019). Basically, the geochemistry of the element depends on both, atmospheric emissions and lithogenic powdery winds. Therefore, the As-Co-Ge-V distribution can be used as a proposed mechanism for possible tracking of anthropogenic poly-metallic enrichments. Arsenic distribution in the study area is lithological dependent from the old volcanism (surroundings of Probishtip-Kratovo).

Distribution of As ranges from 4 to 150 mg/kg, while in the topsoil layer, it reaches 90 mg/kg. Thus, the arsenic can be identified as an anthropogenic tracer in the investigated area. Mapping data extract the anthropogenic enrichment in the middle course of the river basin, where the agriculture activities dominate in the land use. The investigated area is affected with enriched content in arsenic, especially in the area of Pb-Zn mines. Germanium has a specific and unique distribution in the environment. Its geochemistry is also linked to gallium. The minerals of germanium are extremely rare, and the porous germanium is widespread to a considerable extent. Extraction of germanium from waste dust is complicated, not only because of its low concentrations but also because of its amphoteric properties. Germanium contents in the deposited dust from the investigated area reach 2.1 mg/kg. Similar occurrence has been determined for tin as well. Reimann et al. (2012) explains significant correlations in the geochemistry of both elements. Spatial mappings for arsenic, cobalt, germanium and vanadium are presented in Figs. 6.15, 6.16, 6.17, and 6.18.

6.8 Conclusion

The air is intangible, and most of humanity does not think about its importance while inhaling it; however, it is necessary for the existence and functioning of its living world on the planet Earth. With the development of society and technology, man began to engage in production activities that result in the emission of pollutants into the atmosphere. This has significantly begun to change the chemical composition of the planet's ozone layer. Changes in the atmosphere were not noticed until catastrophic pollution began to occur, which took human lives and destroyed flora and fauna. Although there is evidence of efforts to limit human activity that impairs air quality since the sixteenth century, serious efforts in this field were made only in the 1960s, and since then, humanity has become aware of the need to preserve breathing air. Atmospheric deposition poses significant ecological concerns. Dry deposition is characterized by direct transfer of gas phase and particulates from air to ground, vegetation, water bodies and other Earth surfaces.

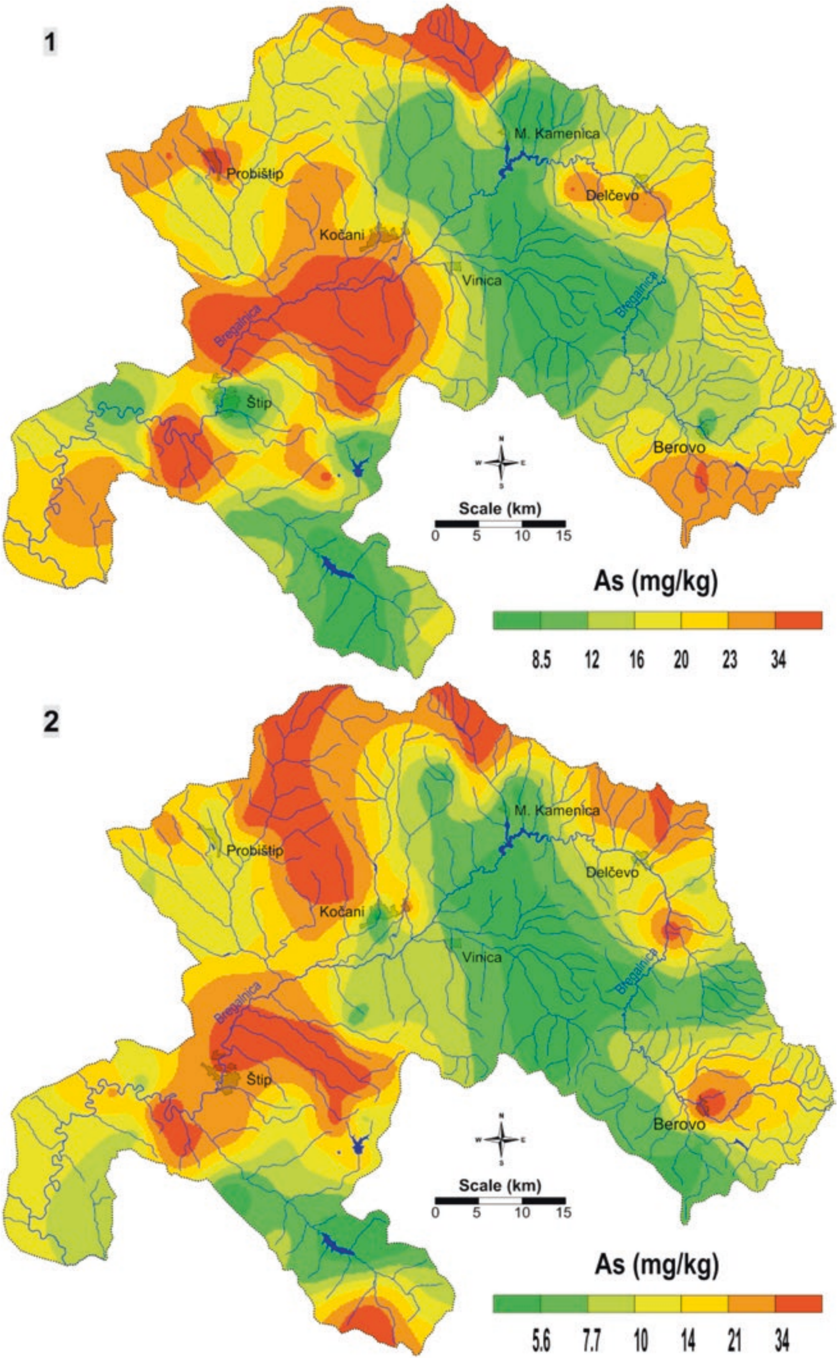

Fig. 6.15 Spatial mapping with *kriging* method for distribution of arsenic in attic dust (1) and soil (2)

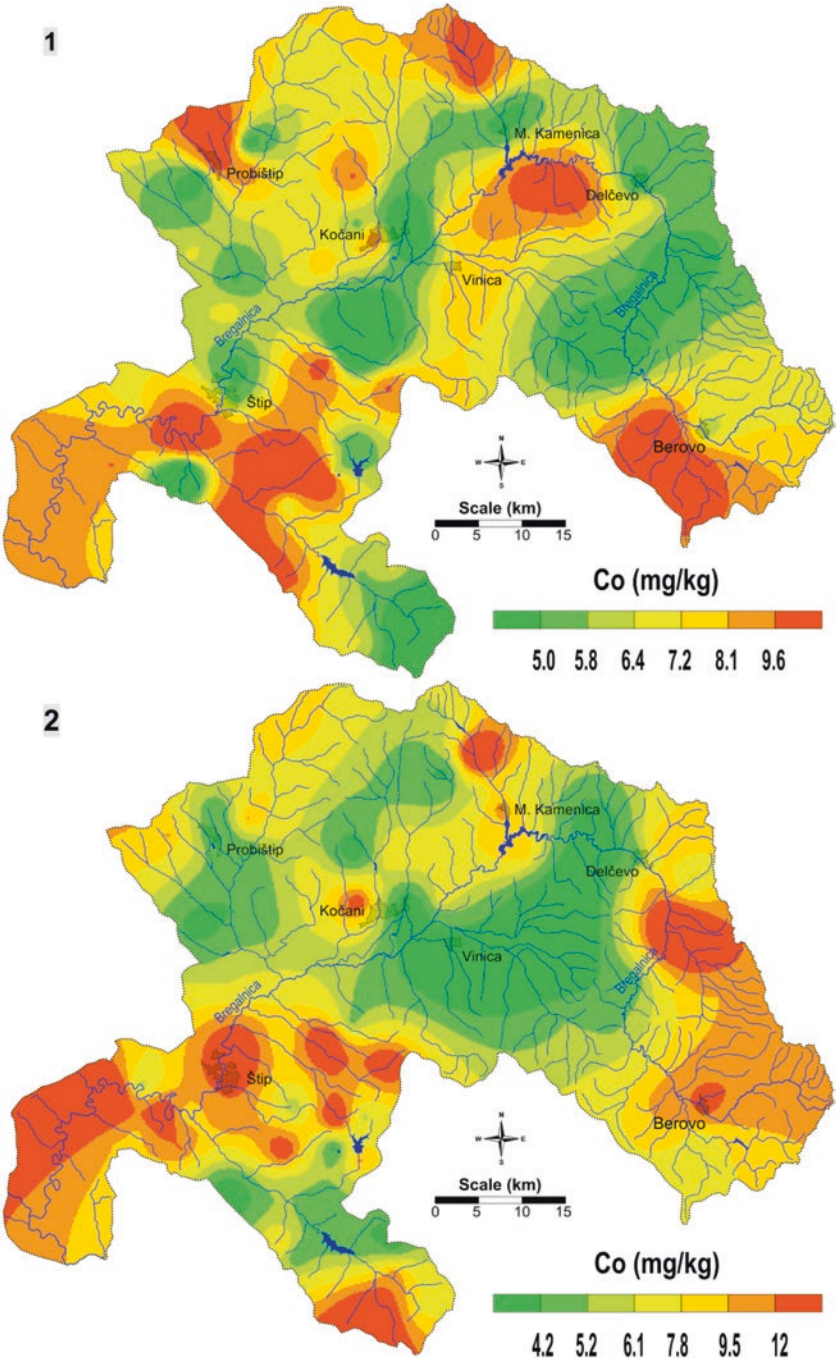

Fig. 6.16 Spatial mapping with *kriging* method, for distribution of cobalt in attic dust (1) and soil (2)

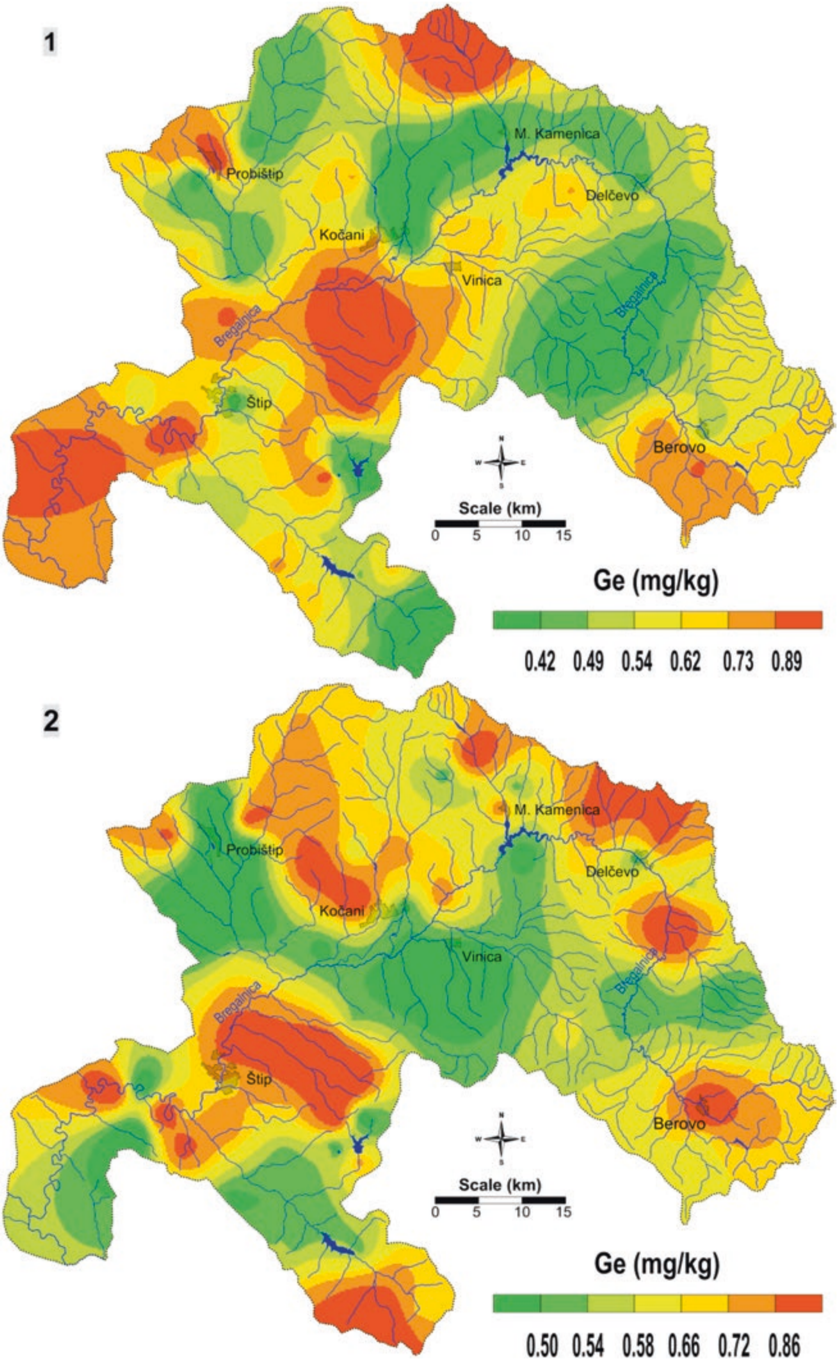

Fig. 6.17 Spatial mapping with *kriging* method, for distribution of germanium in attic dust (1) and soil (2)

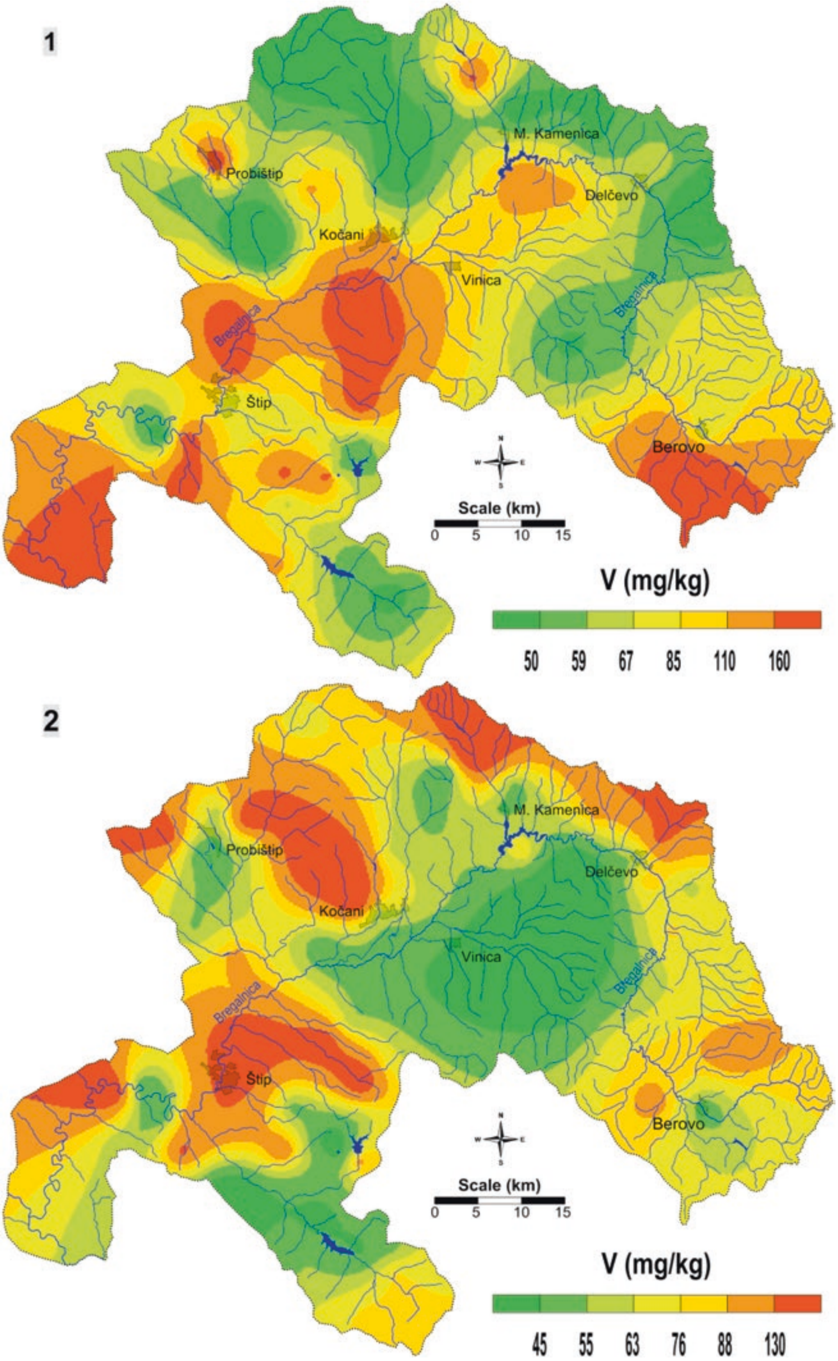

Fig. 6.18 Spatial mapping with *kriging* method, for distribution of vanadium in attic dust (1) and soil (2)

A huge number of toxic substances are continuously emitted into the air, creating unhealthy living arrangements. This “invisible enemy” of the biosphere quietly threatens the life of organisms, including predominantly humans. That is why today a large number of researchers are committed to fast and efficient methodologies for qualitative and quantitative characterization of air and potential emission pollutants. This is especially important for areas where emission sources are not known. The model presented in this research included the characterization of house attic dust in order to determine the long-term deposition of dust emitted from mine and flotation tailings. The applied multivariate analysis in combination with geostatistical models (on spatial visualization of the elemental contents) gave an effective interpretation of the situation with the long-term deposition in the examined area. The applied methodology effectively defined the lithogenic vs. anthropogenic markers in the environment, due to the long-term emissions of potentially toxic metals.

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Chapter 7
Improving Quantitative Analysis of GC-MS for Tracking Potential Contaminants in Groundwater

Biljana Kovacevik, Zoran Zdravkovski, Sasa Mitrev, and Natalija Markova Ruzdik

Abstract The behavior of nonvolatiles benalaxyl, buprofezin, chlorpyrifos, malathion, methomyl, metribuzin, pirimiphos methyl, pyrimethanil, triadimenol, penconazole, and pirimicarb is investigated when increased pressure injection of 10, 20, 30, 40, and 50 psi and vent time of 0.5 and 1.5 min are used during the injection time. The result showed that the peak area obtained during the increased pressure of 50 psi significantly differs from the peak area obtained when the classical hot splitless injection was performed. No significant difference was observed between the investigated vent times. The proposed method using 50 psi for vent time of 0.5 min was validated for benalaxyl, buprofezin, chlorpyrifos, pirimiphos methyl, pyrimethanil, pirimicarb, and triadimenol. The presence of these pesticides was investigated in the groundwater of the investigated area. Pyrimethanil was detected in 3 out of 20 investigated samples in a maximum concentration of 0.3392 μg/l. Chlorpyrifos was detected in only one sample in a concentration of 0.0367 μg/l. Correlation analysis was performed to find a possible relationship between the presence of pesticides and ions which predominantly results from human activities. Positive correlation with sulfates and potassium as well as low Na/Cl ratio suggests that pesticides are probably derived in groundwater leaching from the surface area.

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7.1 Introduction

Before the late 1970s, pesticides were not considered as potential contaminants of groundwater due to a belief that soil acts as a natural filter so they will bind to the soil particles on their way to the groundwater basins or will simply break down in more innocuous substances. The first step in disproving of this belief was the detection of dibromochloropropane (DBCP) in 2000 wells in California and aldicarb in 96 wells in Long Island, in 1979 (*Cohen* et al. 1984). The discovery of groundwater pesticide contamination in subsequent surveys resulted in significant concern about the threat of pesticides as potential groundwater contaminants and establishment of maximum allowable concentrations for pesticides in water for human consumption of 0.1 μg/l for individual and 0.5 μg/l for total pesticides in the Directive (*EC* 1980). These values have been accepted and became statutory limits in many European countries. In the period from 1979 to 1988, around 77 pesticide active materials were discovered in groundwater of 39 states in the USA, attributed mostly to known point sources, misuse, and normal agriculture activities (*US EPA* 1992). In 1985, atrazine and bentazon were detected in groundwater in Germany (*Williams* et al. 1988). One year later, in 1986, Norway reported the presence of dichloropropane (*Williams* et al. 1988). Atrazine, simazine, bentazon, and molinate were detected the same year in Italy and atrazine, simazine, and some phenoxy herbicides in Great Britain (*Williams* et al. 1988). Detailed overview of pesticide detections in European groundwater is given by the European Environment Agency (EEA). As a result of these findings, developed countries started continuous monitoring of pesticides in groundwater that contributed to the introduction of Directive 2000/60/EC by which EU member states are obligated to make a characterization of the chemical composition of the groundwater, including specification of the contributions from human activities, and to provide information to the EEA (*EC* 2000). To reach this aim, Groundwater Directive 2006/118/EC (*EC* 2006) was established and lays down detailed quality criteria for the assessment of groundwater chemical status in Europe. Member states were obliged to establish those threshold values for the first time by 22 December 2008 and to publish them in the Water Framework Directive (WFD) river basin management plans by 22 December 2009. By 2004, 12 EU member states have submitted reports about the presence of pesticides in groundwater (*EEA* 2004). The most frequently detected were herbicides from the group of triazines. Austria reported the presence of desethylatrazine and atrazine in 15% and 10% of investigated wells, respectively, with concentrations greater than MCLs of 0.1 μg/l. In France almost half of the monitored wells were unpolluted, 35% showed contamination potential, and 13% were seriously contaminated. In Germany in 2001, residues of pesticides were observed in 27% of investigated wells from which only 8.5% showed concentrations greater than MCLs. Great Britain in 2000 reported that

9% of investigated wells didn't meet the expectations for unpolluted groundwater. Only Sweden stated that there is no concern about pesticide groundwater pollution on its territory (*EEA* 2004). The presence of pesticides was detected in 24.4% of the total 279 investigated wells during the national survey of pesticides in groundwater in New Zealand performed in 2018 (*Close and Humphries* 2019). Most commonly found were also herbicides from the group of triazines. The most frequently detected was terbuthylazine followed by dieldrin, hydroxyatrazine, atrazine, and terbacil. Bentazone, hexazinone, bromacil, terbacil, desethyl terbuthylazine, 2-hydroxyatrazine, alachlor, picloram, glyphosate, and the total atrazine and its metabolites were found in concentration that exceeds the EU threshold value of 0.1 μg/l.

Triazines are of particular importance when it comes to groundwater pollution due to their high persistence (long half-life), high water solubility, and poor absorption by soil particles. Atrazine and simazine are the most commonly found at concentrations above 0.1 μg/l. Therefore, atrazine and simazine together with alachlor, anthracene, chlorfenvinphos, chlorpyrifos, 1,2-dichloroethane, dichloromethane, diuron, endosulfan, hexachlorobenzene, hexachlorocyclohexane, isoproturon, pentachlorobenzene, pentachlorophenol, trifluralin, trichlorobenzenes, and tributyltin compounds are classified in the priority group of pesticides by the Annex II of the Directive 2008/105/EC set by the European Parliament and the Council of the European Union (*European Parliament* 2008).

Some pesticides other than the most commonly found may also be present in groundwater but due to their low leaching capability and hydrogeoecological conditions of the region are present in undetectable concentrations. The goal of this study was to investigate the presence of nine thermally labile nonvolatile (benalaxyl, buprofezin, chlorpyrifos, malathion, methomyl, metribuzin, pirimiphos methyl, pyrimethanil, and triadimenol) and two thermally stable, nonvolatile pesticides (penconazole and pirimicarb) in groundwater situated under the agriculture land where dripping irrigation is common practice, using GC/MS and liquid-liquid extraction (LLE). To improve the method detection limits and to increase the response of the thermally labile pesticides, an increased pulsed pressure during the injection time was used. The obtained result was statistically processed to find the best injection conditions for their identification and quantification.

7.1.1 Factors Contributing to Pesticide Leaching

Leaching of pesticides occupies the attention of scientists since the first pesticide discovered in groundwater. Several factors are involved in this process. Physical and chemical characteristics of the pesticide, geological, ecological, field conditions, as well as agriculture practices are the most important factors contributing to pesticide leaching. All of these factors act simultaneously and interdependently.

Water solubility is one of the most important features when it comes to pesticide leaching. Highly water-soluble pesticides have greater potential to reach groundwater. Water solubility is related to the soil and water pH which influence pesticide sorption. Organic basic pesticides like s-triazine in low pH will dissociate in cationic compounds and will be adsorbed more by the negatively charged clay or organic matter particles (*Weber* et al. 1969). Acidic pesticides like sulfonylurea herbicides, 2,4-D, dinoseb, etc., exposed in environment several pH units higher than the pesticide dissociation constant expressed as pK_a, will dissociate in anionic compounds which will result in decreased adsorption and higher leachability. Pesticide volatility is also important. When a high volatile pesticide is used by foliar application, there is a high probability that a greater amount will remain in the air. However, if a highly volatile pesticide is applied in the soil, a greater amount will be dissolved in soil pore-trapped water and together with the surface water will reach the aquifers. This process is considered as the most acceptable for leaching of soil fumigants like ethylene dibromide (EDB) and dibromochloropropane (DBCP), found in groundwaters in the USA (*US EPA* 1992). Henry's law constant (k_H) is a major determinant of a pesticide tendency to volatilize from water solution to the air, and thus mostly pesticides with low k_H will persist in soil, surface, and groundwaters.

Numerous research have shown that not less important for pesticide leaching are geological and ecological characteristics of the region. These factors act complexly and simultaneously, and in favorable conditions, pesticides may reach groundwater in several days (*Kordel and Klein* 2006). Differences in ecological and geological characteristics between the regions result in different outcomes. The depth and the type of the aquifer, the porosity of the bedrock, and field conditions like soil type, soil porosity, organic matter content, clay content, and the structure of the soil are important features contributing to pesticide leaching. Soil porosity is directly related to water leakage from the top layer of the soil through the subsoil and bedrock into the aquifers, carrying the dissolved substances. Increased need of irrigation that appears in cultivated soils will increase the amount of water leakage through the soil layers. Good agriculture practice requires careful planning of pesticide application in porous soils. Grouping of soil particles into aggregates influence water leakage, too. Topsoils represented with large aggregates and macropores as a result of some physical processes enable greater leakage of water compared to soils with compact structure. The depth of the aquifer is also important. Shallow groundwaters up to 30 m depth (*Freeze and Cherry* 1979) are more polluted because of the shorter way needed to reach the aquifer which contributes to decreasing the time during which pesticides are prone to degradation and adsorption. *Mehnert* et al. (2005) in their study showed that the concentration of pesticides in shallow groundwater with depth less than 6 m is three times higher than in groundwater with depth from 6 to 15 m. Regarding this it is important to consider the soil characteristics. Sandy and lime soils possess higher permeability compared to clay soils. The tendency of pesticides to leach depends on how strong they will be adsorbed to the soil particles. Strongly adsorbed pesticides despite their high solubility will have lower leaching potential. They will be exposed to the processes of degradation and decomposition for a longer time or will be absorbed by the rooting system of the plants.

Leaching capabilities of weakly adsorbed pesticides will depend on their solubility in water. The sorption forces are a function of pesticide chemical properties, soil type, and the organic matter content. Indicator for the adsorption of pesticides to the soil particles is the adsorptive partitioning coefficient (K_d), calculated as a ratio between the amount of a pesticide adsorbed to the soil and the amount that remains in the water. Higher K_d values indicate the higher capability of the pesticide to be adsorbed to the soil particles, and thus the leaching potential of such pesticides is lower. This coefficient doesn't take into account the organic matter content in the soil which has a great capability to adsorb pesticides in the soil. This limitation was overcome when the organic carbon coefficient (K_{oc}) was introduced, calculated as the ratio between the K_d and the percent of the organic carbon in the soil (*Karickhoff* et al. 1979). Similar behavior to organic matter showed the clay particles in the soil. They adsorb ionic pesticides and trap them on their way to the aquifer exposing them to the processes of degradation and decomposition for a longer time (*Talbert and Fletchal* 1965; *Bailey and White* 1970; *Paim and Lagenbach* 1996).

Worrall (2001) in their study of pesticide leaching conclude that when it comes to highly water-soluble pesticides the behavior is different. Highly water-soluble pesticides will leach very fast especially when high precipitations occur no matter the amount of clay and organic matter content in the soil, the porosity of the soil, or the bedrock. *Weber* et al. (1969) studied the adsorption of s-triazines from aqueous solutions by organic soil colloids at different pH levels from 1 to 5.2 and concluded that maximum adsorption occurs at pH levels close to the pK_a values of the respective compound. Considering all these properties mentioned previously, *Cohen* et al. (1984) set up the important physical and chemical characteristics of a pesticide leacher: water solubility greater than 30 mg/kg, partition coefficient between soil and water (K_d) less than 5 usually less than 1 or 2, soil organic partition coefficient (K_{oc}) less than 300–500, Henry's law constant (k_H) less than 10^{-2} atm^{-1} m^{-3} mol, negatively charged at ambient pH, hydrolysis half-life greater than 25 weeks, photolysis half-life greater than a week, and field dissipation half-life greater than 3 weeks. For the evaluation of pesticide leaching potential, various indices are proposed in the literature. These indices are index-based screening tools that use the physical and chemical properties of the pesticide concerning soil properties. The evaluation is made considering setting threshold values (Table 7.1).

7.1.2 Methods for Determination of Pesticides in Groundwater

The extraction and development of simultaneous multi-residue methods for pesticide determination are a great challenge since the differences in chemical and physical properties like different solubility and volatility and different degrees of polarity and pK_a values result in different behavior during the processes of extraction, clean up, injection, and chromatographic separation. Due to these differences, there is no single universal technique for pesticide identification. On the other hand, low detection limits for groundwater (100 μg/l) set by the EU Commission and the regulatory agencies contribute to the complexity of their identification. According to *Tomlin* (2003), more than 860 pesticide active substances are used in pesticide formulations on the market, belonging to more than 100 substance classes. In general, thermally

Table 7.1 Main indices for evaluation of pesticide leaching potential proposed in the literature

Index	Equation	Interpreted criteria	References
Hamaker's RF (Hamaker's retardation factor)	$\mathrm{RF} = \frac{1}{\left\{1 + (Koc \cdot foc \cdot Pb \cdot \left(\theta^{-0.67} - 1\right)\right\}}$	*0.64–1, high;* *0.35–0.64, moderate; 0.1–0.35, low;* *< 0.1, very low*	*Hamaker* (1975)
Brigg's RF (Briggs's retardation factor)	$\log\left(\frac{1}{\mathrm{RF}} - 1\right) = \log(Kow) + \log(OM) - 1.33$	*0.90–1.0, class 5 (very high); 0.89–0.65, class 4 (high); 0.64–0.35, class 3 (moderate); 0.34–0.10, class 2 (low); 0–0.09, class 1 (very low)*	*Briggs* (1981)
Organic carbon-water partition coefficient	$K_{oc} = \frac{K_d \cdot 100}{\text{Organic matter\%}}$	*0–50 very high; 50–150 high; 150–500 medium; 500–2000 low; 2000–5000 slight; >5000 immobile*	*McCall* et al. (1981)
LEACH (leaching index)	$\mathrm{LEACH} = \frac{WS \cdot DT_{50}}{V_p \cdot K_{DC}}$	*Lower values mean lower leaching potential*	*Laskovski* et al. (1982)
AF (attenuation factor)	$\mathrm{AF} = \exp\left[\frac{-0.693 \cdot d \cdot \mathrm{RF} \cdot \theta_{FC}}{q \cdot t_{1/2}}\right]$	*0 to −1, high;* *−1 to −2, moderate; −2 to −3, low;* *−3 to −4, very low;* *< −4, nonleachable*	*Rao* et al. (1985)
GUS (groundwater ubiquity score)	$\mathrm{GUS} = (\log DT_{50}) \bullet (4 - \log K_{DC})$	>2.8, leachable; 1.8–2.8, intermediate; <1.8, nonleachable	*Gustafson* (1989)
LPI (leaching potential index)	$\mathrm{LPI} = \frac{1000 \cdot DT_{50} \cdot g}{0.693 \cdot L \cdot \mathrm{RF} \cdot Q_{fc}}$	*LPI > 90, very high; LPI = 75–89, high;* *LPI = 50–74, moderate; LPI = 25–49, low;* *LPI = 0–24, very low*	*Meeks and Dean* (1990)
HI (Hornsby index)	$\mathrm{HI} = \left(\frac{Koc}{t_{1/2}}\right) \cdot 10$	*≤10, high;* *≥2000, low*	*Hornsby* (1992)
PLP (pesticide leaching potential index)	$\mathrm{PLP}_{\mathrm{value}} = \frac{R \cdot F \cdot t_{1/2}}{Koc}$ $\mathrm{PLP}_{\mathrm{index}} = (\log \mathrm{PLP}_{\mathrm{value}}) \bullet (14.3) + 57$	*90–100, very high;* *70–89, high;* *50–69, moderate;* *30–49, low;* *0–29, very low*	*Warren and Weber* (1994)

Index	Equation	Interpreted criteria	References
GWCP (groundwater contamination potential)	$\mathrm{GWCP} = \frac{\mathrm{PLP} + \mathrm{CLP}}{2}$	*>150, high;* *75–150, moderate;* *<75, low*	*McLaughlin* et al. (1994)
AFT (log-transformed attenuation factor) AFR(revised attenuation factor)	AFT = In AF/(−0.693) AFR = In AFT + k	*Lower values mean lower leaching potential*	*Li* et al. (1998)
RLP (relative leaching potential index)	$\mathrm{RLP} = \left(\frac{K_{oc}}{DT_{50}}\right) \cdot 10$	*Higher values mean lower leaching potential*	*Warren and Weber* (1994)
LIX (screening leachability index)	$\mathrm{LIX} = \exp\left(-0.693 \cdot \frac{K_{DC}}{DT_{50}}\right)$	*1, high leachable;* *0.1–1, leachable;* *0–0.1, transition;* *0, nonleachable*	*Spadotto* (2002)
LIN (leaching index)	LIN = 0.495 log Kow + 0.518 log Sw− 0.495 log Koc − 0.023 log Vp − 0.452 log K_H	*Lower values mean lower leaching potential*	*Gramatica and Di Guardo* (2002)
M. LEACH (modified LEACH)	$\mathrm{M.LEACH} = \frac{Sw \cdot t_{1/2}}{Koc}$	*Lower values mean lower leaching potential*	*Papa* et al. (2004)
GLI (global leachability index)	GLI = 0.579 LIN + 0.558 GUS + 0.595 M. LEACH	*>1, high;* *−0.5 to 1, medium;* *< −0.5, low*	*Papa* et al. (2004)
DTK (depth, $\mathrm{t}^{1/2}$, K_{OC} model)	$\mathrm{DTK} = e^{-\mathrm{depth}} \cdot \left(a \cdot In\, t_{1/2} - b \cdot In \cdot Koc\right)$	*>0 potential leacher; ≤0 nonleacher*	*Webb* et al. (2008)
VI (volatility index)	$\mathrm{VI} = \frac{200 \cdot k \cdot \theta_{FC}}{d \cdot Pb \cdot \left(\%OM\right)} \cdot \frac{t_{1/2}}{Koc} \cdot F_{DGW}$	*Lower values mean lower leaching potential*	*Gurdak* (2014)
YASGEP-P	YASGEP-P = (0.892) GUS + (0.709) LIX + (0.926) M.LEACH + (0.805) LIN + (0.749) Briggs's RF + (0.844) Hamaker's RF + (0.910) PLP + (−0.860) AFR	*Lower values mean lower leaching potential*	*Akay Demir* et al. (2019)

$t_{1/2}$ half-life (days), Θ volumetric soil water content, *Z or d* depth to groundwater (m), q net groundwater recharge rate (m/day), ρ_b soil bulk density (kg/m^3), *OM* organic matter, S_w water solubility (mg/l), V_P vapor pressure (mm Hg), Θ_{FC} volumetric water content at field capacity, *F* fraction of pesticide reaching the soil during application, K_H Henry constant, K_{ow} octanol/water partition coefficient, K_{oc} organic carbon normalized soil sorption coefficient (ml/g organic carbon), *RF* retardation factor, *V* volatility (bar), f_{oc} organic carbon fraction, *R* rate of pesticide application (kg/ha)

Table 7.2 Review of the GC/MS methods for the determination of pesticides in groundwater

Reference	Extraction method	LOD, µg/l	Investigated pesticides
Silva et al. (2011)	SPME	0.0006–0.024	Atrazine, simazine, alachlor, ethofumesate, chlorpyrifos, desethylatrazine, lindane, α-endosulfan, metolachlor, β-endosulfan, metribuzin, permethrin
Casado et al. (2000)	MLLE	0.04	Carbetamide
Benjamin et al. (2012)	LLE	0.001–0.01	α-Endosulfan, β-endosulfan sulfate, dieldrin, p,p-DDE, p,p-DDT, chlorpyrifos.
Barbash et al. (2001)	SPE	0.001–0.018	Atrazine, cyanazine, prometon, simazine, alachlor, acetochlor, metolachlor
Lary et al. (2014)	LLE	0.09–0.78	α-HCH, α-endosulfan, chlorpyrifos, parathion methyl
Goncalves et al. (2007)	SPME	58–180	Atrazine, desethylatrazine, dimethoate, pendimethalin, endosulfan, endosulfan sulfate, lindane, terbuthylazine, diazinon, propyzamide, metribuzin, alachlor, chlorpyrifos, parathion ethyl, chlorfenvinphos, procymidone, fenamiphos, 4,4-DDE, dieldrin, λ-cyhalothrin, α-cypermethrin, metolachlor
Ghanem et al. (2011)	LLE	1–250	2,4-D, MC, paraquat, atrazine
Shomar et al. (2005)	SPE	0.02–0.05	Atrazine, atrazine desisopropyl, propazine, simazine, triadimenol, DDT, DDE, DDT
Knee et al. (2010)	LLE		Carbaryl, metalaxyl, and metribuzin
Robinson (2001)		0.1	Pirimicarb, carbamate metabolites
US EPA Method 525.3 (2012)	SPE	0.0025–0.047	118 pesticides
Filho et al. (2010)	DI-SPME	0.02–0.3	Trichlorfon, diazinon, methyl parathion, malathion, fenthion, ethyon, bifenhin, permethrin, cypermethrin, imazalil, prochloraz, azoxystrobin, pyraclostrobin, carbofuran, clofentezine, difenoconazole

LOD limit of detection, *SPME* solid-phase microextraction, *SPE* solid-phase extraction, *MLLE* micro liquid-liquid extraction, *LLE* liquid-liquid extraction

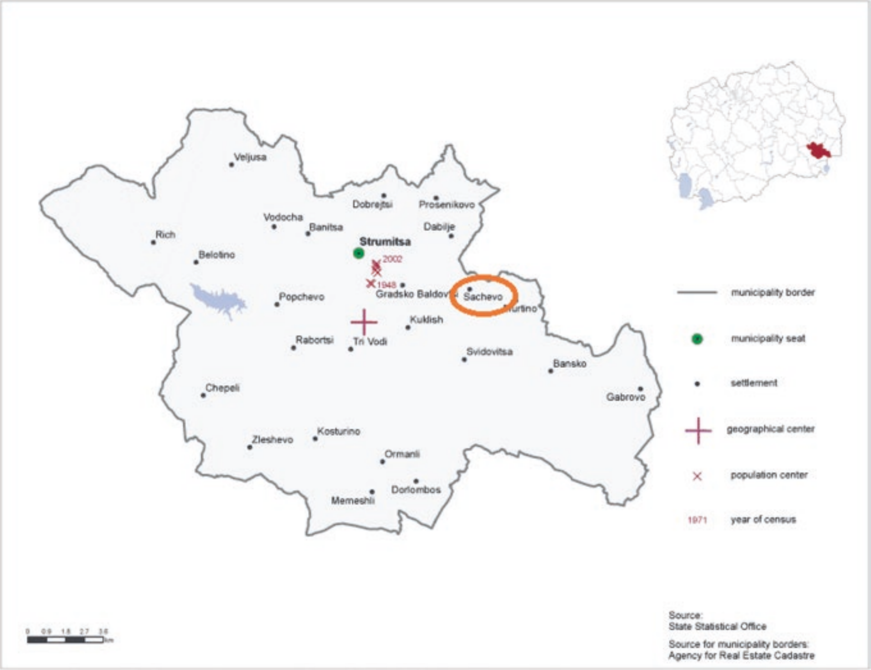
stable pesticides are readily analyzed by gas chromatography (GC) while thermally labile compounds by liquid chromatography (LC). Identification and confirmation are the most accurate when mass spectrometry (MS) analyzers are involved especially when a nontarget analysis is performed. Advances in chromatography contribute to the publication of various multi-residue analytical methods in the literature. Some of GC/MS methods proposed in the literature for pesticide determination in groundwater are given in Table 7.2.

Nonvolatile pesticides, especially those prone to decomposition, thermolysis, or adsorption during their residence time into the injection port, are difficult to be analyzed by GC using hot splitless injection. Many authors find that increased pressure during the injection may significantly reduce this, due to reduced contact with the hot, active inlet surface (*Stan and Goebel* 1984; *Grob* 1988; *Stan and Müller* 1988; *Husmann* et al. 1990; *Wylie and Uchiyama* 1996; *Kovacevik* et al. 2016). The evidence of the available literature data suggests that no general rules exist for deriving optimal parameters for pulsed splitless injection. Literature data is still in lack of information due to a large number of components that differ in their physical and chemical properties, the various number of combinations, and instrument conditions.

7.2 Materials and Methods

7.2.1 Characteristics of the Investigated Area and Sampling

Groundwater samples were collected from the vulnerable area of the village Sachevo (41°25′15.16″N; 22°41′58.87″E), 7 km from the town of Strumica, located in the southeast part of the Republic of North Macedonia (Fig. 7.1). Groundwater is classified as highly vulnerable regarding the type of aquifer classified as young alluvium aquifer, poor technical condition, no flow regulation in the convey structures,



Source:
State Statistical Office

Source for municipality borders:
Agency for Real Estate Cadastre

Fig. 7.1 The region of Strumica and the location of groundwater sampling for pesticide analysis, the settlement of Sachevo and unregulated use (*Popovska and Geshovska* 2014). Twenty groundwater samples were collected from the existing boreholes on the field occupied with the greenhouse production of early vegetables mostly from Solanaceae, Brassicaceae, and Cucurbitaceae families. Groundwater is a primary source of irrigation water, exploited mainly through domestic boreholes located on the field. Dripping irrigation systems are common practice in the region. Twenty groundwater samples were collected in the period 2014–2015. Groundwater sampling was done according to EPA guidelines (*Johnston* 2007). For that purpose water was left to flow around 10 minutes until stabilization of ECw. For pesticide analysis, an amount of 1 l was collected in clean glass bottles and kept at 4 °C and analyzed within 48 hours. For determination of major ions and heavy metals, amount of 2 l was collected in glass bottles and preserved with H_2SO_4 for determination of calcium bicarbonate ($CaCO_3$), ammonium ions (NH_4^+), nitrates (NO_3^-), nitrites (NO_2^-), and phosphates (PO_4^{3-}) and HNO_3 for determination of heavy metals (HM) and sulfates (SO_4^{2-}).

7.2.2 Analysis of Physical and Chemical Characteristics of Groundwater

A total number of 25 physical and chemical properties of collected groundwater samples were determined including depth (*d*), temperature (*T*), hydrogen ion activity (pH), conductivity (ECw) and hardness ($CaCO_3$), major ions (Mg^+, Na^+, K^+, Ca^{2+}, Cl^-, NO_3^-, NO_2^-, NH_4^+, HCO_3^-, SO_4^{2-}, and PO_4^{3-}), heavy metals (Cd, Cu, Cr, Mn, Zn, Fe, and Pb), and the trace mineral boron (B). Groundwater depth and temperature were determined in situ; pH was determined using the pH meter HANNA HI 2211-01 according to the standard procedure prescribed by the manufacturer. Conductivity was determined by the conductometer Jenway 4520. Major ions like Cl^-, NH_4^+, NO_3^-, NO_2^-, SO_4^{2-}, and PO_4^{2-} and total hardness expressed as $CaCO_3$ were determined by a colorimetric method using UV-Vis spectrophotometer, type Jenway 6715, according to the US EPA Methods 325.2 (1978), 350.2 (1974), 352.1 (1971), 354.1 (1971), 365.3 (1978), and 130.1 (1971). Heavy metals were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500 CX). The equipment was linearly calibrated for the detector response in the range of 1 to 100 µg/l, using a certified standard solution (Sigma ICP multielement standard solution). Linearity was checked after every ten samples. Accuracy has been tested by analyzing a certified reference material, NIST SRM 1643 c “trace elements in water.” Bias ranged from 2% to 7%. Precision, expressed as intermediate precision, was better than 10% for all analyzed elements.

7.2.3 Analysis of Pesticides

7.2.3.1 GC Conditions

Separation conditions were the same in all experiments. The oven temperature ramped from 60 °C for 2 min to 150 °C with an increment of 25 °C/min for 0 min, ramped to 200 °C with an increment of 3 °C/min for 0 min, and ramped to 280 °C with an increment of 20 °C/min for 10 min, for a total running time of 41.8 min. The temperature of the injector was 250 °C, the temperature of the MS quadrupole was 150 °C, and the injection volume was 1 μl. Classical hot splitless injection versus splitless injection using increased pressure from 10 to 50 psi at flow to split vent for 0.5 min and 1.5 min were evaluated for a better response of the analyte considering the peak area and the response factor. For that purpose, each injection was conducted in three repetitions, and the mean value was used to calculate the percent of improvement. The extraction of pesticides from the groundwater was made by continuous liquid-liquid extraction (LLE) using 1 l of water and 40 ml of dichloromethane (DCM) added in three portions of 20 ml, 10 ml, and 10 ml. First, the solvent was added into the glass bottles to dissolve the pesticides adsorbed on a glass wall. Then it was transferred into the separatory funnel of 2 l in which previously the groundwater sample of 1 l was added. Water was salted out with 10% of sodium chloride (NaCl), and then the water and the solvent were shaken for 2 min and allowed to stand for 10 min to separate the phases. After the extraction, the solvent was evaporated under the flow of nitrogen and collected in a 2 ml vial using DCM. The solvent is evaporated again under the nitrogen flow, and the volume of 100 μl hexane was added in the vial, vortexed for 10 s, and collected in a vial insert. For recovery analysis water was spiked with pesticide standards in a concentration of 0.1 and 0.5 μg/l. Limit of detection (LOD) and limit of quantification (LOQ) were determined using regression ANOVA analysis. Quantification was done using triphenyl phosphate (TPP) as an internal standard in a concentration of 0.05 ng/μl.

7.2.3.2 Solvents and Standards

Certified chemical standards (purity 95–99%) and solvents with HPLC grade were obtained from Sigma-Aldrich. Pesticide stock solutions and working standards were prepared in acetone. Calibration curves were prepared in the concentration range of 0.1–3.2 ng/μl.

7.2.3.3 Apparatus and Instrumentation

Pesticide analyses were performed on gas chromatograph Agilent 6890N coupled to a mass spectrometer and equipped with JAS UNIS split/splitless injector with electronic pressure control and Agilent 7683B Series Autoinjector. Glass liner type (JAS

90323L) with deactivated borosilicate wool and single restriction, 3 mm ID and 750 µl maximum volume capacity, was used in all experiments. For the separation of analytes, DB-5 ms Supelco column (30 m × 0.25 mm × 0.25 µm) was used with helium as a carrier gas and a total flow of 1 ml/min. The mass spectrometer was operated in electron ionization mode with ionizing energy of 70 eV, MS quadrupole temperature 150 °C, and solvent delay of 4.3 min. The analysis was performed in the selected ion monitoring mode (SIM) based on one target and two qualifier ions. The method was validated evaluating the linearity, recoveries, LOD, LOQ, and precision.

7.2.3.4 Data Processing and Statistical Analysis

The efficiency of the extraction (E_e) was calculated according to *Mol* et al. (1995):

$$E = \left(1 - \frac{1}{1 + Ke\left(\frac{Ve}{Vs}\right)}\right)^n$$

where *Ke* is the partition coefficient of the pesticide, *Ve* is the volume of the solvent, Vs is the volume of the water, and *n* is the number of extractions.

A descriptive analysis was performed to characterize groundwater samples. Two-factorial ANOVA was used to evaluate the significant differences between the response factors obtained from the analyte responses when using different pulse pressures and different vent times. Post hoc Tukey HSD test for dependent samples was performed to determine the significant differences between the groups. One-factorial ANOVA was used to evaluate differences between groundwater samples regarding their pollution, and correlation analysis was performed to evaluate possible relationships between pesticides and ions derived from the surface agriculture activities.

7.3 Results and Discussion

7.3.1 Increased Pressure Injection

Low volatile and thermally label compounds are difficult to be analyzed by gas chromatography; therefore many laboratories are trying to find various solutions to minimize the negative impact of the hot inlet port and improve their detectability so they can be analyzed simultaneously together with the volatile and thermostable compounds. One of the advances in gas chromatography is the possibility to use increased pressure during the time of injection which decreases the time of the residence of analytes into the injection port. The technique is known as "pulsed pressure injection" and it was first introduced by *Wylie* et al. (1991), who investigated the impact of the pulsed pressure on sample discrimination and allowable injection volumes in the analysis performed on a gas chromatograph coupled to atomic emission detector (AED) and GC equipped with flame ionization detector (FID). The authors also investigated the effect of pulsed pressure on the decomposition of two labile pesticides endrin and 4,4′–DDT and the carbamate pesticide, carbaryl, using GC coupled to atomic emission (AED), electron capture (ECD), and flame ionization detection. The result showed a substantial reduction of analyte decomposition for aldrin, 4,4′-DDT, and modest improvements for carbaryl. After that many researchers investigated the behavior of various compounds when pulsed pressure injection is used. *Godula* et al. (1999) in their study come to the conclusion that for achieving good responses for all analytes especially the low volatiles, the pressure-time should not exceed 1 min and the intensity of 60 psi. The authors also noticed that the injection of sample volumes greater than 1 μl causes peak distortion unless a retention gap is used. *Wylie* et al. (1991, 1992) observed that increased pressure during the injection significantly reduce the decomposition of aldrin, DDT, and aldicarb. Next, the authors investigate six organophosphorus pesticides and conclude that the pulsed pressure injection dramatically improves the recovery of notoriously difficult pesticide acephate (*Wylie and Uchiyama* 1996). The behavior of nine thermally labile nonvolatile (benalaxyl, buprofezin, chlorpyrifos, malathion, methomyl, metribuzin, pirimiphos methyl, pyrimethanil, and triadimenol) and two thermally stable, nonvolatile pesticides (penconazole and pirimicarb) when increased pressure of 10 psi, 20 psi, 30 psi, 40 psi, and 50 psi and vent time of 0.5 and 1.5 min are used during the time of injection is investigated in this study.

The obtained results as peak response and response factors were compared with the peak response and the response factor when the classical hot splitless injection is used and expressed as a percentage of improvement. The physical and chemical properties of investigated pesticides are given in Table 7.3. The obtained

Table 7.3 Chemical and physical properties of investigated pesticides

	Boiling point / °C	Degradation point / °C	Vapor pressure at 20 °C/ mPa	Sw	pK_a	Log P	GUS
Benalaxyl	463	250	0.572	28.6	nd	3.54	0.51
Buprofezin	273	177	0.46	0.042	nd	4.93	0.46
Chlorpyrifos	200	170	1.43	1.05	nd	4.7	0.15
Malathion	ni	174	3.1	148	nd	2.75	0.6
Methomyl	144	192	0.72	55,000	nd	ni	2.20
Metribuzin	132	230	0.121	10,700	1.3	1.75	2.57
Penconazole	436	Stabile	0.336	73	1.51	3.72	1.36
Pirimiphos methyl	ni	162	0.002	11	4.3	4.2	2.82
Pirimicarb	325	Stabile	0.43	3100	4.4	1.7	2.73
Triadimenol	465 ± 55	270	0.0005	72	nd	3.18	3.75

Ni no information, *nd* no dissociation, *Sw* water solubility at 20 °C

Table 7.4 Improvement of pesticide response (%) evaluated as peak area when pulsed pressure of 10 psi, 20 psi, 30 psi, 40 psi, and 50 psi is used versus classical hot splitless injection

	0.5 min vent time					*1.5 min vent time*				
Pesticide	10 psi	20 psi	30 psi	40 psi	50 psi	10 psi	20 psi	30 psi	40 psi	50 psi
Benalaxyl	29	162	217	286	375	85	110	128	165	152
Buprofezin	23	57	103	97	114	26	65	90	101	109
Chlorpyrifos	26	114	224	297	349	45	136	227	276	366
Malathion	29	204	431	660	1043	28	237	434	670	1048
Methomyl	78	254	462	707	855	75	238	492	712	944
Metribuzin	15	61	104	131	168	25	79	115	145	176
Penconazole	30	80	91	120	137	30	98	137	119	192
P. methyl	35	104	172	227	251	47	124	163	215	259
Pirimicarb	30	77	119	139	168	48	90	124	156	180
Pyrimethanil	19	52	104	118	148	30	59	110	120	152
Triadimenol	14	52	87	133	128	18	64	105	112	168

Table 7.5 Improvement of the response factor (RF) expressed in percentage when pulsed pressure of 10 psi, 20 psi, 30 psi, 40 psi, and 50 psi is used versus classical hot splitless injection

	0.5 min vent time					*1.5 min vent time*				
Pesticide	10 psi	20 psi	30 psi	40 psi	50 psi	10 psi	20 psi	30 psi	40 psi	50 psi
Benalaxyl	107	133	120	126	153	128	107	82	85	69
Buprofezin	102	79	77	64	69	87	84	68	64	57
Chlorpyrifos	104	109	123	130	145	100	120	118	120	128
Malathion	106	154	201	248	366	89	171	192	246	314
Methomyl	147	179	213	264	308	121	172	213	259	286
Metribuzin	96	82	77	76	86	86	91	77	78	75
Penconazole	108	91	73	72	76	90	101	85	70	80
P. methyl	112	103	103	107	113	102	114	95	100	98
Pirimicarb	108	90	83	78	86	103	96	80	82	77
Pyrimethanil	98	77	77	71	80	90	81	76	70	69
Triadimenol	94	77	71	76	73	81	83	74	68	73

improvements for both investigated vent times were expressed in percentage and are given in Table 7.4. All investigated pesticides showed peak improvement. The most dramatic improvement was observed for malathion and methomyl suggesting that these two pesticides probably are most affected by the performances of the injector (Table 7.5; Fig. 7.2). Two-way ANOVA with replication was conducted to evaluate the differences between the response improvements of analytes when the vent time of 0.5 min and 1.5 min was used and between the groups when increased injection pressure (10 psi, 20 psi, 30 psi, 40 psi, 50 psi) was used. The obtained results showed significant differences for the response improvement when different pressures are used ($p < 0.05$), but no significant differences were observed between the responses of the pesticides when different vent time is used, and no significant interaction exists between the vent time and the pulse pressure (Table 7.6). This outcome

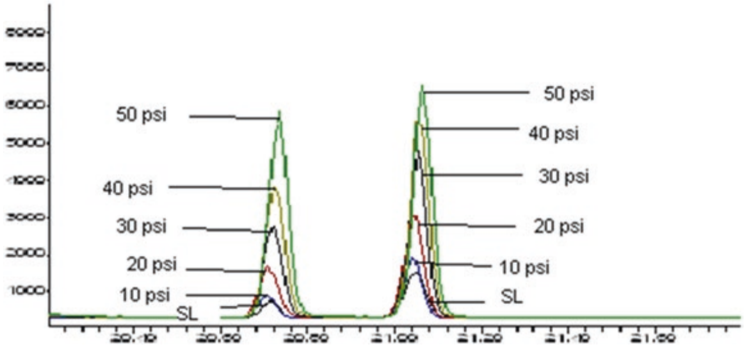


Fig. 7.2 Peak improvement of malathion (20.736 min) and chlorpyrifos (21.064 min) when increased pressure and vent time of 0.5 min are used

Table 7.6 Two-way ANOVA with replication, between the groups for 0.5 min and 1.5 min vent time, and treatment of 10–50 psi, $\alpha = 0.05$

Source of variation	SS	df	MS	F	*p*-value	F crit
Sample	$1.98e^{11}$	4	$4.94e^{10}$	7.84	$1.35e^{-5}$	2.45
Columns	78,684,239	1	78,684,239	0.012	0.91	3.93
Interaction	$1.05e^{9}$	4	$2.62e^{8}$	0.04	0.99	2.45
Within	$6.93e^{11}$	110	$6.3e^{9}$			
Total	$8.91e^{11}$	119				

SS sum of squares, *df* degree of freedom, *MS* mean square, *F* distribution, *F crit* critical distribution

suggests that the influence of the vent time when nonvolatile compounds are subject to the pulsed pressure injection when single trapped glass wool liner is used is negligible. To evaluate the differences between the analyte responses when hot splitless and increased pressure of 10 psi, 20 psi, 30 psi, 40 psi, and 50 psi are used during the time of injection, statistically significant ANOVA was followed up by the Tukey HSD post hoc tests for dependent samples (*Tukey* 1949). The result showed that the response of the analytes at pulsed pressure of 50 psi significantly differs from the responses of other pressures used. Statistical results suggest that the best improvement for the response will be achieved if the pressure of 50 psi is used (Table 7.7). Taking all together and considering the improvements for the calculated response factors (Table 7.5), it is assumed that increased injection pressure of 50 psi for vent time of 0.5 min will significantly affect the response improvement of the investigated pesticides and most likely will improve the limit of detection in the method.

Based on the obtained results, simultaneous ion monitoring (SIM) method was created for quantification of investigated pesticides using increased pressure of 50 psi and vent time of 0.5 min (Table 7.8). LOD and LOQ were obtained using regression ANOVA analysis. The obtained LODs were in the range of 0.001–0.167 ng/µl. The method was used to investigate pesticides in groundwater. For that purpose, continuous LLE was performed using methylene chloride as a solvent. Calculated

Table 7.7 Tukey HSD post hoc test between the groups ($q = 2.93$; critical value 47,465)

Comparison	Absolute mean difference
10 psi vs 20 psi	25,085
10 psi vs 30 psi	43,273
10 psi vs 40 psi	**53,756**
10 psi vs 50 psi	**60,064**
20 psi vs 30 psi	18,188
20 psi vs 40 psi	28,670
20 psi vs 50 psi	**85,150**
30 psi vs 40 psi	10,482
30 psi vs 50 psi	**103,338**
40 psi vs 50 psi	**113,821**

Significant values are marked with bold
q studentized range distribution

extraction efficiencies showed satisfactory results for all investigated pesticides except for methomyl and metribuzin. The recovery study performed from a spiked water sample at 0.1 μg/l showed satisfied recovery in the range from 68 to 117% using LLE with methylene chloride (Figs. 7.3, 7.4 and 7.5). LODs were in the range of 0.001–0.388 ng/μl; LOQs were in the range of 0.005–1.17 ng/μl. RSD was lower than 10% for all compounds (Table 7.8). The proposed method was validated for benalaxyl, buprofezin, chlorpyrifos, pirimiphos methyl, pyrimethanil, pirimicarb, and triadimenol and used for identification of pesticides in groundwater.

7.3.2 Groundwater Quality

7.3.2.1 Pesticides in Groundwater Samples

Pesticides can reach water-bearing aquifers below ground from applications onto crop fields, seepage of contaminated surface water, accidental spills and leaks, improper disposal, and even through injection waste material into wells.

In this study 15% of investigated groundwater samples show the presence of pesticides. Pyrimethanil and chlorpyrifos were identified in 3 out of 20 investigated groundwater samples in a maximum concentration of 0.3392 μg/l (Table 7.9). Chlorpyrifos was widely used in agriculture for the control of soil and foliar pests before it was banned in the European Union in January 2020. It is an organophosphate volatile compound that has low aqueous solubility and low soil mobility. Based on these chemical properties, it is considered that chlorpyrifos possesses a low risk of leaching to groundwater. Despite this, it is found in groundwaters worldwide like Pakistan (*Tariq* et al. 2007), New Zealand (*Close and Skinner* 2012), Argentina (*Loewy* et al. 2011), Brazil (*Bortoluzzi* et al. 2007), Turkey (*Tuncel* et al. 2008), Australia (*Wightwick and Allinson* 2007), Spain (*Teijon* et al. 2010), Ireland (*Estévez* et al. 2012), Greece (*Vryzas* et al. 2012), Portugal (*Silva* et al. 2011), the

Table 7.8 Method parameters when pulse pressure injection of 50 psi is used

	Quantifier ion	Qualifier ions	E_e %	t_s Min	LOD ng/µl	LOQ ng/µl	RSD %	R %	R^2	Regression equation
Pyrimethanil	198	199	97.04	16.40	0.048	0.146	3.14	102	0.9993	$y = 0.0751 \cdot x - 0.002$
Pirimicarb	166	72, 238	99.6	17.42	0.167	0.507	2.89	116	0.9921	$y = 0.0535 \cdot x - 0.0016$
Pirimiphos methyl	290	276, 305	98.76	20.12	0.001	0.005	3.47	68	0.9940	$y = 0.0374 \cdot x - 0.0013$
Chlorpyrifos	197	97, 199	99.99	21.06	0.388	1.17	6.34	68	0.9923	$y = 0.1114 \cdot x - 0.0116$
Triadimenol	112	168, 128	93.14	23.82	0.112	0.341	5.00	92	0.9964	$y = 0.0166 \cdot x - 0.0003$
Buprofezin	105	106, 172	99.96	26.07	0.003	0.008	2.92	65	0.9987	$y = 0.0698 \cdot x - 0.0003$
Benalaxyl	148	91, 206	98.58	27.99	0.21	0.65	4.52	104	0.9871	$y = 0.046 \cdot x + 0.0017$

Ee extraction efficiency, t_s retention time, *LOD* limit of detection, *LOQ* limit of quantification, *RSD* relative standard deviation, *R* reproducibility, R^2 linear regression

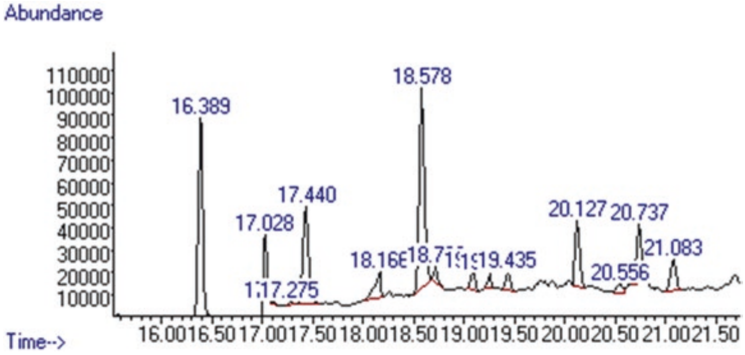


Fig. 7.3 Real groundwater sample spiked with pyrimethanil (16.389 min), pirimicarb (17.440 min), pirimiphos methyl (20.127 min), and chlorpyrifos (21.083 min) at a concentration of 0.1 μg/l

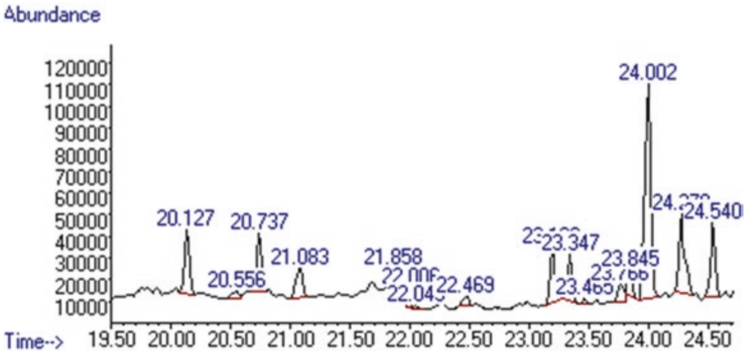


Fig. 7.4 Real groundwater sample spiked with triadimenol (24.002 min) at a concentration of 0.1 μl/l

USA (*Gilliom* et al. 2006), etc. In groundwater of the investigated area, chlorpyrifos was identified in one sample in a concentration of 0.0367 μg/l. Pyrimethanil is a fungicide from the chemical group of anilinopyrimidine compounds, widely used in vegetable production for control of gray mold, powdery mildew, and downy mildew. It is moderately soluble in water, nonvolatile, weak-based pesticide, moderately persistent in the soil with the ability to leach in groundwater and stay stable at pH 5–9. Literature data about the presence of pyrimethanil in groundwater are scarce. It is found in 1 of 12 analyzed groundwater samples in the agriculture area occupied with potato production in the USA in concentration of 4.1 ng/l (*Reilly* et al. 2012). The available information about the toxicity of pyrimethanil in groundwater provides evidence that this pesticide can be a decisive factor in the habitat selection process of many species. It disturbs the environmental quality and is potentially toxic for many aquatic species, affecting their survival, reproduction, feeding, and growth (*Araújo* et al. 2015).

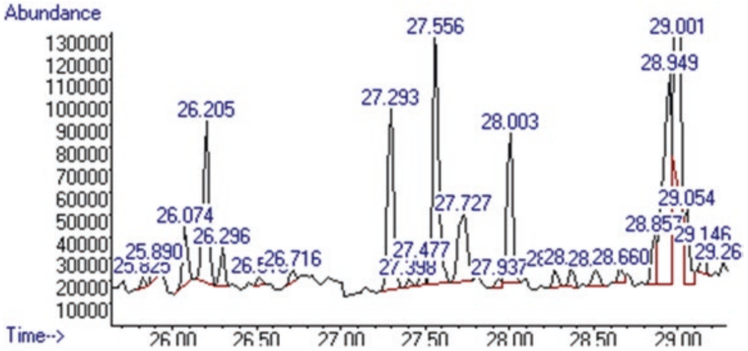


Fig. 7.5 Real groundwater sample spiked with buprofezin (26.074 min), benalaxyl (28.003 min) at a concentration of 0.1 μg/l, and triphenyl phosphate (29.001 min) at a concentration of 0.5 μg/l

Table 7.9 Pesticides found in groundwater samples from the investigated region

Coordinates	*d*	*C* (μg/l)	Pesticides
41°25′432″N 022°41′648″E	24 m	0.0151 0.0367	Pyrimethanil Chlorpyrifos
41°25′022″N 022°41′891″E	23 m	0.0136	Pyrimethanil
41°25′785″N 022°46′067″E	21 m	0.3392	Pyrimethanil

Descriptive analysis of investigated quality parameters (Table 7.10) suggests that no significant pollution of groundwater in the investigated region is present. Groundwater is mostly shallow and slightly alkaline and the major ions are bicarbonates. Conventional agriculture production on the soil surface is the main indicator of the vulnerability of groundwater regarding pollution with nitrates, potassium, and phosphates leaching from the fertilizers as well as pesticides applied for plant protection. Many authors have found a correlation between the presence of pesticides in groundwater and nitrates leaching from fertilizers (*Burow* et al. 1998; *Istok* et al. 1993). One-way ANOVA performed to investigate possible differences between the samples regarding investigated variables showed no significant difference between the investigated samples (Table 7.11). Therefore the correlation analysis was performed to find a possible relationship between the presence of pesticides and ions which predominantly results from human activities. No correlation was observed between the amount of pesticides and nitrates in groundwater, but the positive correlation between sulfates and potassium as well as the low Na/Cl ratio suggests that pesticides are probably derived in groundwater leaching from the surface area (Table 7.12). Low nitrate content in groundwater despite its great use in the investigated region and wider environment suggests the presence of some processes contributing to this occurrence. Based on the geochemical properties of the investigated aquifer (young alluvium aquifer); pH 7–8; high Fe, Mn, and HCO_3^- content; as well as low SO_4^{2-} and NO_3^- content, it is assumed that there is a reducing

Table 7.10 Descriptive statistics for investigated parameters of groundwater in the area of Sachevo

	Unit	X	X(BC)	Me	Min	Max	P25	P75	σ	CV	A(BC)	B(BC)
D	M	54	4	26	6	97	21	92	38	70	−0.3	−1.2
T	^{0}C	16.5	3	16.7	15	17.2	16.2	17	0.6	3.6	−1.4	2.5
pH	–	8.2	2.3	8.3	7.6	8.6	8.2	8.3	0.2	2.8	−1.4	2.8
ECw	µS/cm	5.1	1.7	5	2.7	11.8	4.4	5.2	2	39	1.2	3.6
Mg^{2+}	mg/l	5.6	1.6	3.9	2.7	29.5	3.4	4.3	6.3	113	2.8	9.5
Na^+	mg/l	11.5	2.6	11	5.8	27	8.9	12.4	5	44	0.7	1.6
K^+	mg/l	25.4	2.2	5.4	3.1	336	4.8	7.6	80	315	3.6	14
Ca^{2+}	mg/l	34.4	3.9	35.4	17.9	47	29	37.5	7.2	21	−1.2	2
$CaCO_3$	mg/l	304	6.8	308	131	516	274	334	83	27	−0.8	2.9
HCO_3^-	mg/l	370	7.1	376	160	629	333	407	102	27	−0.8	2.9
Cl^-	mg/l	13.7	2.7	11	9	50	11	13.3	9.4	69	3.4	13
NO_3^-	mg/l	1.4	0.2	1.3	0.5	3	1	1.6	0.6	44	−0.3	1
NH_4^+	mg/l	3	−0.2	0.8	0.03	31.6	0.4	2.2	7.5	252	0.3	1.4
NO_2^-	mg/l	0.03	−3.3	0.03	0.03	0.03	0.03	0.03	0.0	0.0	0.0	0.0
SO_4^{2-}	mg/l	6.9	1.1	2.5	0.8	77	2.5	2.5	18.1	265	3.3	13
PO_4^{3-}	mg/l	0.4	−1.6	0.2	0.03	3.6	0.1	0.4	0.9	192	0.6	0.8
B	µg/l	7.5	1.4	4.5	1.4	55	3.6	6.7	11.7	157	1.1	4.5
Cd	µg/l	3.5	0.05	0.05	0.5	0.05	0.05	0.1	140	4.4	19	0.1
Cu	µg/l	0.2	−1.7	0.2	0.1	0.6	0.2	0.25	0.1	44	0.5	2.8
Cr	µg/l	0.3	−1.4	0.3	0.3	1.3	0.3	0.25	0.2	75	4.4	19
Mn	µg/l	615	4	745	1.6	1257	337	901	329	54	−4	17
Zn	µg/l	17.6	1.8	5	2.3	89	3.3	16,4	25.7	146	0.9	−0.6
Fe	µg/l	163	3.3	69	21	760	43	222	214	131	0.5	−0.4
Pb	µg/l	0.3	−1.9	0.2	0.06	1.3	0.1	0.3	0.3	113	0.7	1.7

X mean of not transformed values, *X(BC)* mean of the Box-Cox transformed values, *Me* median, *Min* minimum, *Max* maximum, *P25* 25th percentile, *P75* 75th percentile, σ standard deviation, *CV* coefficient of variance, *A (BC)* skewness of Box-Cox transformed values, *B (BC)* kurtosis of Box-Cox transformed values, *d* depth of the borehole, *ECw* conductivity

Table 7.11 One-way ANOVA F-test for investigated groundwater samples regarding anthropogenic pollution indicators

Source	DF	SS	MS	F-stat	p-value
Between groups	16	45729.23	2858.08	0.3003	0.9962
Within groups	238	2265070.55	9517.10		
Total	254	2310799.78			

environment in the investigated groundwater (*Chapelle* et al. 2008; *Jurgens* et al. 2009; *McMahon and Chapelle* 2008; *Kovacevik* et al. 2016) and that low NO_3^- content found in groundwater probably results from the processes of denitrification that appears in such environment. This goes in addition to the significant positive correlation between pH and NO_3^- found in the statistical analysis and no correlation between the amount of pesticides and ions that originate from the fertilizers prone to reduction like NO_3^- and PO_4^{3-}.

Table 7.12 Correlation analysis of the physical and chemical properties of groundwater in the investigated region

	Pesticides	*d*	pH	ECw	NO_3^-	NH_4^+	SO_4^{2-}	PO_4^{3-}	K^+
Pesticides	1								
d	0.25	1							
pH	0.39	0.03	1						
ECw	**0.51**	0.15	−0.03	1					
NO_3^-	−0.08	0.03	**−0.5**	0.48	1				
NH_4^+	**−0.57**	−0.11	**−0.72**	−0.04	**0.70**	1			
SO_4^{2-}	**0.76**	−0.30	0.38	**0.53**	−0.18	−0.48	1		
PO_4^{3-}	0.26	−0.28	−0.26	0.19	0.30	0.40	0.42	1	
K^+	**0.69**	−0.06	0.32	**0.74**	−0.07	−0.39	**0.88**	0.31	1

Significant correlations are marked with bold

7.4 Conclusion

Liquid-liquid extraction with low amount of dichloromethane followed by the concentration of the sample of 10,000 times and increased pressure injection in the range of 10–50 psi for vent time of 0.5 and 1.5 min was investigated in this study for 9 thermally labile nonvolatile (benalaxyl, buprofezin, chlorpyrifos, malathion, methomyl, metribuzin, pirimiphos methyl, pyrimethanil, and triadimenol) and two thermally stable, nonvolatile pesticides (penconazole and pirimicarb). The result showed that the pressure of 50 psi significantly improves the peak response of the investigated pesticides and decreases the level of decomposition of the thermally labile pesticides. No statistically significant influence was observed regarding the duration of the vent time, and no significant interaction exists between the vent time and the pulse pressure. Two-factorial ANOVA followed by the post hoc Turkey test can be a useful tool in distinguishing statistically significant differences between the improvements in different method performances in instrumental analysis. Acceptable recovery can be obtained even if lower amounts of DCM like 40 ml is added in portions during the process of pesticide extraction like pyrimethanil, pirimicarb, pirimiphos methyl, chlorpyrifos, triadimenol, buprofezin, and benalaxyl from groundwater. If no significant groundwater pollution is observed, extracts from groundwater can be concentrated up to 10,000 times to lower the detection limits. The negative side of this is the need of highly competent and skilled staff. Pyrimethanil and chlorpyrifos were detected in maximum concentration of 0.3392 and 0.0367 μg/l, respectively. The obtained result indicates that pyrimethanil is likely to be found in the groundwater under the agriculture area where it is applied if deposits like alluvial sediment sands, gravels, clays, and sandy clays are present. Despite its low toxicity, it should be considered as a potential groundwater contaminant and included in the multi-residual methods more often since it can disturb the natural balance of the groundwater.

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Chapter 8
Groundwater Pollution Under the Intensive Agriculture Production

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Abstract Groundwater quality situated under the intensive agriculture production of the Strumica region, Republic of North Macedonia, is investigated. Carbonate rock watering plays a significant role in groundwater chemistry. Nitrate concentrations greater than 50 mg/l were observed in 14% of investigated samples with a maximum concentration of 284 mg/l (med 3 mg/l). Almost 33% of Mn levels were above the levels of public health concern (>50 μg/l). Arsenic was found in elevated concentrations (>10 μg/l) in almost 35% of investigated groundwater samples, and 16% had concentration greater than 50 μg/l (max 177 μg/l). It is assumed that the reductive dissolution is a major mechanism by which arsenic is released into the groundwater. Statistical analysis shows significant differences regarding NH_4^+, PO_4^{3-}, Fe, Mn, As, Ba, Ti, and Zn concentrations between shallow and deep groundwater. Factor analysis revealed four significant factors. F1 (Cl^-, SO_4^{2-}, Na, K, and B) associates the most affected ions by rainwater leaching. F2 (HCO_3^-, Ca, As, and Mg) makes an association between ions that arise from the hydrogeochemical reactions. F3 (NO_3^-, NO_2^-, Mg, and Cu) associates ions affected by the reduction processes, while F5 (NH_4^+, PO_4^{3-}, and Fe) associates ions which are related to the use of fertilizers.

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Keywords Strumica river basin · Nitrate · Arsenic · ANOVA · Factor analysis

8.1 Introduction

Most of the water found on earth (97.3%) is saline distributed mostly in oceans and seas. The remaining water is freshwater that exists as ice in the polar caps and glaciers from which only 0.61% is freshwater found in lakes, rivers, streams, and groundwater. Groundwater presents the most valuable natural resource of freshwater in the world. Around 95% of the total freshwater available for man on the earth comes from the aquifers. According to a European Commission survey (2008), almost 75% of the world population depends on groundwater as a primary source for drinking water. About 60% of the total amount of groundwater is used for irrigation of agricultural land, and the remaining 40% of the exploitation is almost equally distributed to domestic and industrial sectors (Vrba and van der Gun 2004). Increasing population, industrialization, and agriculture practices are the main reasons for the uncontrollable exploitation of groundwater in many areas in the world, especially in undeveloped countries. After a certain period, this may lead to a decrease in groundwater quality and quantity. Restoring groundwater quality is a very difficult, expensive, and time-consuming process. Considering this and the small amount of freshwater available on the earth, it is of prime importance to assess groundwater quality in each region as well as to identify possible sources of pollution threatening to decrease its quality to implement on time measures and practices for protection and preservation of its quality.

The quality of groundwater situated under agriculture land is a function of hydrogeological factors such as the depth of the type of the aquifer, hydrological properties of soil, and the amount, timing, and location of aquifer recharge as well as a function of contaminant factors. Groundwater contaminants are categorized as pointed and nonpointed sources of pollution. Point sources are usually industrial and manufacturing facilities, oil production and refining facilities, wastewater plants, landfills, leaking underground storage tanks, septic tanks, spills, accidents, etc. Agricultural activities, seawater intrusion, urban runoff, pipeline networks, acid-mine drainage, oilfield brine injection, dairies, and feedlots are usually recognized as a nonpoint source of pollution (AWWA 2003).

Natural processes like water-rock interaction and topographic composition are the main contributors to the hydrogeochemical characteristics of groundwater. The most common constituents in groundwater regarding its chemical quality are cations like calcium, magnesium, iron, potassium, manganese, and sodium and anions like bicarbonate (HCO_3^-), chloride (Cl^-), fluoride (F^-), nitrate (NO_3^-), silica (SiO_2), and sulfate (SO_4^{2-}). Other constituents present which are less common but still very important when accessing the chemical quality of groundwater are boron, arsenic, lead, selenium, copper, zinc, barium, nitrite (NO_2^-), and the gases oxygen, carbon dioxide, methane, and hydrogen sulfide (Table 8.1) (Hem 1985). Groundwater

Table 8.1 Major and minor chemical constituents in groundwater

Major constituents		Less common constituents	
Cations	Anions	Minor and trace elements and gasses	
Calcium (Ca)	Bicarbonate (HCO_3)	Boron (B)	Barium (Ba)
Magnesium (Mg)	Chloride (Cl)	Arsenic (As)	Nitrite (NO_2)
Iron (Fe)	Fluoride (F)	Lead (Pb)	Oxygen (O_2)
Potassium (K)	Nitrate (NO_3)	Selenium (Se)	Carbon dioxide (CO_2)
Manganese (Mn)	Silica (SiO_2)	Copper (Cu)	Methane (CH_4)
Sodium (Na)	Sulfate (SO_4)	Zinc (Zn)	Hydrogen sulfide (H_2S)

deterioration due to naturally occurring toxic elements is recognized worldwide: Asia (Mukherjee et al. 2006; Luo et al. 2018; Chen et al. 1994; Rahman et al. 2005), South America (Bundschuh et al. 2012), North America (Nordstrom 2002; Welch et al. 1988; Mueller et al. 2001; Ayotte et al. 2003; Haque et al. 2008; Mumford et al. 2012), and Europe (Gurzau and Pop 2012; Ravenscroft et al. 2009). Sources of groundwater pollution due to anthropogenic activities like agricultural, industrial, and domestic use are also identified in many parts of the world (Naseh et al. 2018; Li and Gao 2019; Strebel et al. 1989; Luo et al. 2018; Bouwer 1987; Bouwer et al. 1983). Groundwater situated under intensive agriculture production is vulnerable to pollution that leaches from the applied fertilizers and plant protection products. The small amount of freshwater available on the earth imposes the importance of assessing groundwater quality in each region and identifying possible sources of pollution. The Strumica region is one of the most intensive agriculture areas in the Republic of North Macedonia which heavily depends on groundwater exploitation. The uncontrolled use of chemical fertilizers and lack of sewage systems as well as the improper drilling activities and waste disposal practices as well as the wood and the canning industry which are developed in the investigated region are the main contributors which may lead to deterioration of groundwater quality. Despite that, there is still a lack of information about the groundwater quality assessment in this region. Therefore, the main objectives of this study were to evaluate groundwater quality in terms of identifying the major hydrogeochemical processes and sources of natural and anthropogenic pollution in the groundwater of the Strumica region which anticipates the potential health effects on the region's inhabitants and the possible harmful effects arising from its exploitation.

8.2 Materials and Methods

8.2.1 Investigated Area

The region of Strumica is located in the southeastern part of the Republic of North Macedonia, situated on the Balkan Peninsula, and occupies around 943 km^2 (Fig. 8.1). The age of the lithozone of the Strumica region belongs to the Upper

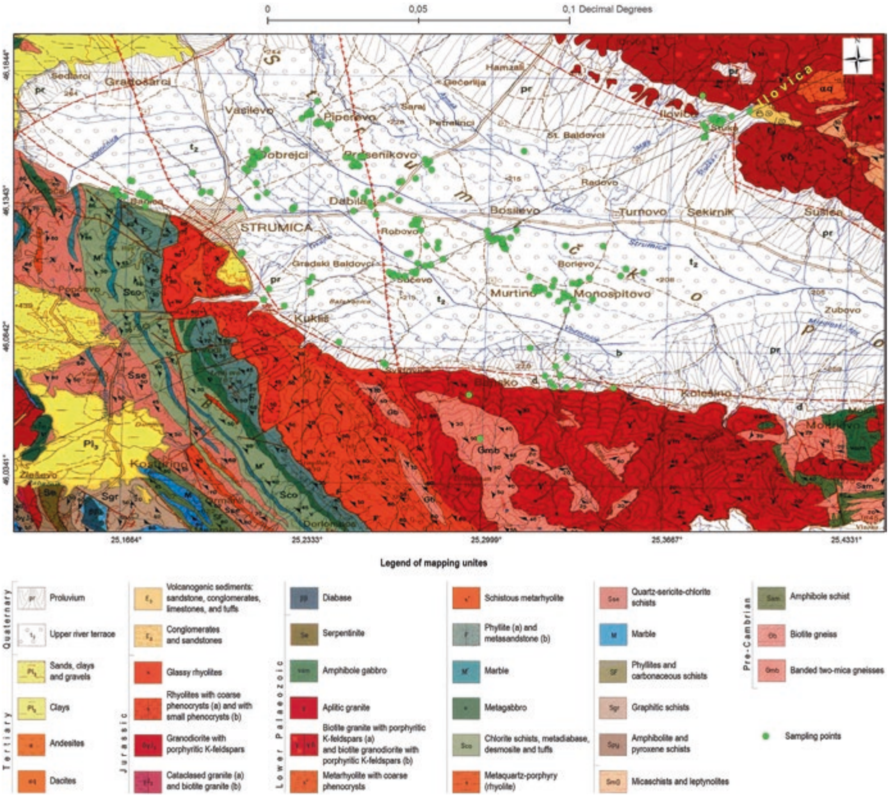

Fig. 8.1 Geological map of the Strumica region and sampling points

Eocene. The discovered thickness of the basal lithozone ranges from 20 to 50 m (Rakicevik and Pendzerkoski 1973). The region is characterized by intensive agriculture production since the 1950s when cotton was the main cultivated crop for the existence of the domestic population and since 1980 when cotton production was replaced by early vegetable production which contributes to the development of the food care industry. The wood industry is another also developed industry in the region. The region is rich in hydrogeothermal water which according to Gorgieva et al. (2000) belongs in the hydrogeothermal systems in the fractured granites of Paleozoic or Mesozoic age. Hot springs and boreholes with different temperatures are present within small distances mostly in the village of Bansko. The maximum measured temperature is 73 °C, and the predicted maximum temperature is 120 °C (Gorgieva 1989). The reservoir in the granites lies under thick tertiary sediments. Abundant mine with copper and gold deposits is present in the village of Ilovica located in a northwest-southeast striking tertiary magmatic arc that covers large areas of Macedonia, Serbia, Central Romania, southern Bulgaria, northern Greece, and Eastern Turkey (Carter 2008). Almost 60% of the land is represented by forest, whereas 30% is covered with arable land. Vegetable production especially crops from the families Cucurbitaceae, Solanaceae, and Brassicaceae is the main agricultural product since the 1980s.

Groundwater of the Strumica/Strumeshnitsa river basin belongs to the Petrich valley aquifer shared by the Republic of North Macedonia and Bulgaria. The aquifer is made up of Pliocene, predominantly, and Quaternary lake sediments and alluvial sands, gravels, clays, and sandy clays (UNECE 2011). The Strumica river is transboundary tributary to the Struma/Strymonas river which source is in western Bulgaria (Vitosha Mountain, south of Sofia) and ends in the Aegean Sea (Strymonikos Gulf – Greece) (Fig. 8.1).

The intensive agriculture production makes this area vulnerable regarding fertilizer and pesticide application as well as sewage and manure leaching. The average air temperature and precipitation are 12.7 °C and 604 mm, respectively. According to Ivanova and Ambarkova (2015), a compact groundwater type with water level under pressure is present (artesian well at a depth of 180–160 m) in the region and the well capacity of 10–20 l/s. Gorgieva et al. (2000) classified the groundwater in the investigated region as highly vulnerable regarding the poor technical condition, no flow regulation in the convey structures, unregulated use, and the decrease of precipitation. The main use of groundwater in the Strumica region is for irrigation and only small part for water supply and industry.

8.2.2 Sampling and Analysis

8.2.2.1 Groundwater Sampling

A total number of 185 groundwater samples were collected from existing boreholes on agriculture land during April, May, and June 2014 and 2015 (Fig. 8.1). Samples were collected according to the EPA guidelines (Johnston 2007). For that purpose, groundwater was left to flow until the conductivity stabilization and collected in clean glass bottles which were transferred in 2 hours, preserved, and kept on 4 °C until the analysis.

8.2.2.2 Chemical Analysis

Groundwater was analyzed for the quantity of major cations (Ca, Mg, Fe, K, Mn, Na), anions (CO_3, HCO_3, Cl, NO_3, SO_4), heavy metals, and trace elements (As, Al, B, Be, Cu, Cr, Co, Cd, Li, Mg, Mo, Ni, Pb, Ti, V, Zn). Anions like chlorine, carbonate, and bicarbonate were analyzed by volumetric methods. Sulfate (SO_4^{2-}), nitrate (NO_3^-), nitrite (NO_2^-), and ammonia (NH_4^+) were determined by the colorimetric method using spectrophotometer type JENWAY 6715, UV-Vis (US EPA Methods 375.4 1978; US EPA Methods 352.1 1971; US EPA Methods 354.1 1971; US EPA Methods 350.2 1974). pH is measured by pH meter HANNA HI 2211-01, and electrical conductivity is measured using the conductometer JENWAY 4520, in situ.

The total oxidation state of arsenic (As), magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), phosphorus (P), manganese (Mn), copper (Cu), nickel (Ni), cobalt (Co), iron (Fe), zinc (Zn) and lead (Pb) was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500 CX). The equipment was linearly calibrated from 1 to 100 μg/l, using a certified standard solution (Sigma ICP Multielement Standard Solution). Linearity was checked after every ten samples. Accuracy has been tested by analyzing a certified reference material, NIST SRM 1643c "trace elements in water." Bias ranged from 2% to 10%.

8.2.2.3 Statistical Analysis

Descriptive statistics analysis was used to perform analysis of data, including mean, median, maximum and minimums, 25th and 75th percentile, standard deviation, variance, and Box-Cox transformed values of mean, skewness, and kurtosis for shallow and deep groundwater. Piper diagram was used for the identification of chemical processes in groundwater using the statistical program Grapher 16.2.354 (2020). Since the quality of groundwater can change at different depths even in the same aquifer, differences between the quality of shallow and deep groundwater were investigated using Welch's t-test for unequal variances and unequal sample sizes. The power of the analysis was performed calculating the type II error. Factor analysis was performed to investigate the relationship between investigated ions using statistical program Statistica, version 10 (StatSoft. Inc. 2011). Varimax normalized type of rotation and multiple R-squared method were used for the extraction of the loadings.

8.3 Results and Discussion

8.3.1 Groundwater Chemistry

The average temperature of the groundwater was 16 °C during sampling. pH values varied from slightly acidic to alkaline (6.84–8.67) with a median value of 7.86. Almost 12.4% of investigated samples were classified as very hard groundwater with total hardness greater than 300 mg/l $CaCO_3$, 36% were hard (total hardness 150–300), 42.5% were moderately hard (total hardness 75–150), and only 9.1% were classified as soft water (0–75) suitable for domestic use.

Based on the borehole depth and the pollution vulnerability, groundwater in the investigated region was classified into two categories: (1) shallow groundwater (6–70 m) where almost 73% of investigated boreholes belong and (2) deep groundwater (72–130 m) where the rest 27% investigated boreholes belong. Literature data showed that groundwater is generally supplied by precipitation, irrigation, infiltration, reservoir seepage, canal seepage, or lateral recharge (Luo et al. 2018). In the investigated area, both categories of groundwater have discharge that is dominated by artificial exploitation.

8.3.1.1 Major Components

Descriptive analysis of major components (Tables 8.2 and 8.3) showed variations in the wide range of concentrations regarding major groundwater constituents. HCO_3^- values varied between 0.05 and 1116 mg/l, SO_4^{2-} values varied between 0.5 and 300 mg/l, Cl^- varied between 7.37 and 614 mg/l, and Ca^{2+} and K^+ values also varied greatly between 7.43 and 411 mg/l and 1.15 and 354 mg/l, respectively. The range is also wide for Mn and Fe which values varied between 0.25 and 3329 mg/l and 0.03 and 3166 mg/l, respectively. This outcome suggests that groundwater in the Strumica differs considerably in the major component concentrations. To investigate if there are significant differences between element concentrations in shallow and deep groundwater, Welch's t-test was performed for normal and Box-Cox transformed values. The result showed significant differences between shallow and deep groundwater for Cl, NO_3^-, NO_2^-, SO_4^{2-}, PO_4^{3-}, Ca, Fe, and Mg when normal values were processed and between Cl, NO_2^-, NH_4^+, SO_4^{2-}, PO_4^{3-}, Fe, Mg, K, and Mn when Box-Cox transformed values were processed (Table 8.4). The power analysis showed a high percentage of accuracy (>95%) only for NH_4^+, PO_4^{3-}, Fe, and Mn when Box-Cox normalized values are processed. Therefore, future statistical analyses are performed on normalized values.

Environmental and agroecological characteristics of the Strumica region indicate high contamination potential of groundwater with nitrate. Despite that, the investigation showed no significant pollution in the majority of investigated samples. Almost 50% showed nitrate concentrations of less than 3 mg/l. Concentrations above 10 mg/l were found in almost 35% of groundwater from which around 14% have concentrations greater than 50 mg/l (max. 284 mg/l). Highly polluted groundwater samples were noticed mostly in shallow groundwater. Nitrate concentration greater than 10 mg/l can cause methemoglobinemia (blue baby syndrome), an often fatal disease in infants. Water with nitrate concentrations greater than 10 mg/l should not be used in infant feeding, by pregnant women or by nursing mothers (US Environmental Protection Agency 2002). The extensive analysis for threshold background concentrations of NO_3^- in groundwater conducted in many different geographic locations showed that concentrations of 2–3 mg/l are usually attributable to natural conditions (Mueller et al. 1995; Burow et al. 1998). Higher concentrations are usually associated with leakage from agriculture fertilizers and sewage manures. Nitrate is soluble in water and can easily pass through soil to the groundwater table and persist in groundwater for decades accumulating at high levels. Research from different regions in the world about nitrate leaching provides evidence for factors that contribute to groundwater vulnerability. Shallow unconfined aquifers associated with agricultural systems as well as unconsolidated aquifers such as the alluvial type of aquifer are most susceptible to nitrate contamination. Groundwater in many agricultural regions in the world was found to be polluted with NO_3^- (Strebel

Table 8.2 Statistics summary of major ions resulting from the descriptive analysis of groundwater samples collected from the Strumica region

	Unit	Mn	Mn(BC)	Md	Min	Max	P_{25}	P_{75}	S	CV	A(BC)	B(BC)
				Shallow groundwater (n = 138)								
d	m	20	3.4	18	4.5	70	15	21.5	10	49.5	0.04	1.9
pH	–	7.9	125	7.8	6.8	8.6	7.5	8.3	0.4	5.6	−0.06	−1.2
ECw	mmho/cm	5.3	2.1	5.2	1.2	17.5	3.5	6.3	2.7	50.6	−0.00	0.2
HCO_3^-	mg/l	277	6.5	299	0.05	751	128	420	173	62.4	−2.6	6
NO_3^-	mg/l	27.8	2.4	3.7	0.1	284	1.1	34.3	51.6	186	0.6	−0.9
NO_2^-	mg/l	0.03	−3.1	0.03	<0.05	0.5	0.03	0.03	0.04	142	7.3	54
NH_4^+	mg/l	0.6	−2.1	0.06	<0.05	56	0.03	0.3	4.8	743	1.9	6.8
PO_4^{3-}	mg/l	0.4	−1.6	0.2	0.07	0.3	0.07	0.3	0.6	175	0.6	0.5
SO_4^{2-}	mg/l	30.2	3.4	21	0.8	300	11.7	31	40	132	−0.06	0.4
Cl^-	mg/l	48.5	4.2	40	7.4	614	20.7	54.5	60	124	0.5	1.5
Na	mg/l	6.5	1.9	5.7	1.4	27	3.6	7.7	4.4	68	0.4	0.2
Ca	mg/l	57	4.6	44.5	7.4	411	28.4	79.6	44	77	0.2	0.4
Mg	mg/l	16.5	2.9	12.3	1.1	96	7.4	21.3	13.4	81	0.01	−0.1
K	µg/l	14.6	2.2	6.5	1.2	354	4	8.9	42	289	1.7	5.5
Mn	µg/l	480	5.1	20.8	<0.5	3329	4	860	672	140	0.1	0.4
Fe	µg/l	163	5.4	57.8	<0.05	3166	50	111	365	224	−0.4	0.4
				Deep groundwater (n = 53)								
d	m	91	5.5	90	18.5	130	80	96	−3.5	20.3	17.9	19.7
pH	–	7.8	9.2	7.7	7	8.7	7.4	8.2	0.3	−1.1	0.5	5.8
ECw	mmho/cm	3.8	3.4	3.9	1.5	6.4	2.6	5	0.2	−0.8	1.3	32.8
HCO_3^-	mg/l	253	7.1	244	79	419	171	335	−0.4	−0.7	99	39.2

	Unit	Mn	Mn(BC)	Md	Min	Max	P_{25}	P_{75}	S	CV	A(BC)	B(BC)
NO_3^-	mg/l	12.3	1.5	2	0.2	77.5	1	19.2	0.5	−1.2	18.4	149
NO_2^-	mg/l	2.6	−2.2	0.03	<0.05	35.8	<0.05	<0.05	2.2	3.1	7.3	285
NH_4^+	mg/l	2.3	−1.0	0.2	<0.05	31.6	<0.05	2.6	0.6	−0.8	5.4	236
PO_4^{3-}	mg/l	1	−0.8	0.4	<0.05	7.8	0.2	0.7	0.8	0.03	1.8	168
SO_4^{2-}	mg/l	9.3	1.7	<0.05	2	167	2.5	8.4	1.8	4.1	23	246
Cl	mg/l	13.2	2.7	11	4.2	81	9.6	13	2	7.2	11	85
Na	mg/l	8	2.2	6.8	2.6	36	5.5	9.2	0.9	2.3	5.1	63
Ca	mg/l	35	4.1	31.7	14.6	76.5	23	43	0.1	−0.7	13.9	40
Mg	mg/l	5.5	1.7	4.8	1.2	12	3.5	7.2	−0.3	0.1	2.6	48
K	µg/l	5.0	1.6	4	2.2	27.3	2.9	5	1.9	5	4	84
Mn	µg/l	409	16.3	371	2.4	2369	225	506	361	88	−0.3	2.6
Fe	µg/l	337	14	161	<0.05	2502	82	501	414	123	0.4	0.5

Mn mean of not transformed values, *Mn(BC)* mean of the Box-Cox transformed values, *Md* median, *Min* minimum, *Max* maximum, *P25* 25th percentile, *P75* 75th percentile, *S* standard deviation, *CV* coefficient of variance, *A* skewness, *B* kurtosis, *d* depth of the borehole, *ECw* conductivity

Table 8.3 Statistics summary of minor constituents resulting from the descriptive analysis of groundwater samples collected from the Strumica region (concentration given in μg/l)

	Me	Me(BC)	Md	Min	Max	P_{25}	P_{75}	S	CV	A(BC)	B(BC)
			Shallow groundwater (n = 138)								
As	15	0.5	1	0.1	172	0.6	5.6	35	233	0.7	−0.5
Ba	26	4.5	26	1.3	128	14.5	31.3	19	72	0.06	1.2
Mn	480	3	21	0.3	3329	4.1	860	672	140	−0.3	−1.1
Ni	3.5	0.4	2.8	0.3	21.6	1.3	5.1	3	80	−0.5	−0.3
Cu	1.4	0.02	1.1	0.1	21.5	0.7	1.8	2	135	−0.6	1.4
Zn	38	2.4	12	2.3	1156	6.3	26.4	124	323	0.9	0.8
Mo	23	2.2	10.7	0.5	166	5.6	24	30	129	−0.2	0.3
Pb	1	−0.6	0.5	0.06	16.3	0.3	0.9	2	186	0.6	0.7
B	13	1.6	6.3	0.03	136	4.2	16.7	18	135	−2.5	7.6
Al	9	1.5	5	0.6	209	3.1	7.2	23	258	0.5	2.6
Ti	184	3.9	152	9.4	1036	69	278	153	83	−0.7	−0.4
V	0.8	−0.8	0.3	0.3	5.2	0.3	1	1	114	0.8	−0.9
Li	5.8	0.9	2.6	0.5	73	0.5	7.5	9	150	−0.03	−1.2
Be	0.4	−1.2	0.3	0.3	2.5	0.3	0.3	0.5	118	2.5	4.5
Cr	1	−0.6	0.6	0.06	6	0.3	1.2	1	112	0.02	−0.4
Co	0.4	−1.3	0.3	0.01	2.1	0.3	0.4	0.4	96	−1.5	9.2
Cd	0.06	−3.4	0.05	0.05	0.50	0.5	0.05	0.05	82	8	68
			Deep groundwater (n = 53)								
As	37.7	2.5	15.2	0.8	177	7.6	65.4	41.7	111	−0.7	−0.1
Ba	41.3	14.1	43	2.8	99	25	54.1	23.2	54	−0.06	−0.1
Mn	409	4.5	371	2.4	2369	2253	506	361	88	−2.8	10
Ni	2.9	0.7	2.3	0.5	10	1.2	3.6	2.2	77	−0.3	−0.6
Cu	1.2	−0.2	0.8	0.2	6.1	0.6	1.2	1.1	96	−0.1	−0.1
Zn	78	2.6	16	2.6	1371	7.3	49.2	227	290	0.6	0.3
Mo	10	1.6	5.4	1	74	2.9	10.7	14	138	0.2	−0.5
Pb	0.6	−0.9	0.4	0.1	5.1	0.3	0.8	0.7	119	0.2	0.4
B	11.7	1.3	7.6	<0.05	90	2.2	15	15	127	−1.6	3
Al	7.4	1.4	5.9	0.4	66	1.9	7.9	9.7	131	−0.4	−0.3
Ti	133	3.9	130	16.9	432	80.3	153	71	54	−0.9	2.8
V	0.6	−1.2	0.3	<0.5	7.6	0.3	0.25	1.4	239	3.4	11.7
Li	6.8	0.8	2.2	<0.5	86	0.5	6.	13.2	193	0.2	−0.9
Be	0.4	−1.3	0.3	<0.5	1.3	0.3	0.3	0.3	89	2.4	3.9
Cr	0.4	−1.2	0.3	0.1	2.5	0.3	0.3	0.4	97	0.8	1.1
Co	0.3	−1.5	0.3	0.02	1.6	0.3	0.3	0.2	80	−1.4	9.2
Cd	0.1	−3.3	<0.1	<0.1	1.8	0.05	0.05	0.3	294	7.3	53

Me mean of not transformed values, *Me(BC)* mean of the Box-Cox transformed values, *Md* median, *Min* minimum, *Max* maximum, *P25* 25th percentile, *P75* 75th percentile, *S* standard deviation, *CV* coefficient of variance, *A* skewness, *B* kurtosis, *d* depth of the borehole, *ECw* conductivity

Table 8.4 Comparative analysis of element concentrations in shallow ($n = 139$) and deep ($n = 53$) groundwater (Welch's t-test for unequal variances) for normal and Box-Cox transformed values

	Normal values					Box-Cox transformed values				
	df	Tstat	Tcrit	P	Power analysis %	df	Tstat	Tcrit	P	Power analysis %
				Major constituents						
HCO_3^-	101	0.27	1.98	0.7889	2.43	154	−0.27	1.98	0.7860	10.37
Cl^-	167	4.75	1.97	$4.41e^{-06}$	0.00	157	6.60	1.98	$5.88e^{-10}$	0.00
NO_3^-	188	3.38	1.97	0.0009	0.00	111	1.82	1.98	0.0708	0.00
NO_2^-	51	−2.53	2.01	0.0147	81.72	52	−2.69	2.01	0.0094	86.80
NH_4^+	82	−1.95	1.99	0.0551	72.14	67	−3.16	1.99	0.0023	96.37
SO_4^{2-}	130	3.62	1.97	0.0004	0.00	111	7.04	1.98	$1.71e^{-10}$	0.00
PO_4^{3-}	56	−2.84	2.00	0.0063	90.02	81	−4.17	1.99	$7.46e^{-05}$	99.91
Ca	189	4.93	1.97	$1.78\ e^{-6}$	0.00	162	1.77	1.97	0.08	0.00
Fe	85	−2.64	1.99	0.0099	91.96	59	−5.20	2.00	$2.64e^{-06}$	99.99
Mg	174	8.76	1.97	$1.67e^{-15}$	0.00	161	7.67	1.97	$1.57e^{-12}$	0.00
K	153	2.51	1.98	0.0132	0.00	169	3.66	1.97	0.0003	0.00
Mn	52	−0.99	2.01	0.3262	25.67	184	−9.28	1.97	$4.73e^{-17}$	100
Na	71	−2.12	1.99	0.0372	75.54	140	0.92	1.98	0.3610	0.00
				Minor constituents						
As	82	−3.45	1.99	0.0009	98.87	114	−9.26	1.98	$1.47e^{-15}$	100
Al	189	0.69	1.97	0.4928	0.20	139	1.00	1.98	0.32	0
B	112	0.64	1.98	0.5212	0.69	67	1.22	2.00	0.225	0.16
Ba	77	−4.72	1.99	0.00001	99.98	82	−7.14	1.99	$3.38e^{-10}$	99.99
Cu	157	1.13	1.98	0.2590	0.06	86	1.69	1.99	0.09	0.02
Mo	182	4.05	1.97	0.00008	0.00	89	3.49	1.99	0.0007	6.42
Ni	117	1.55	1.98	0.1249	0.02	79	0.36	1.99	0.72	2.04
Pb	190	2.21	1.97	0.0282	0.00	115	1.83	1.98	0.07	0.00
Ti	183	3.11	1.97	0.0022	0.00	137	−5.79	1.98	$4.67\ e^{-8}$	99.99
Zn	64	−1.22	2.00	0.2267	35.73	71	−3.05	1.99	0.003	95.13
Li	68	−0.68	2.00	0.4960	18.36	88	−0.40	1.99	0.69	11.48

et al. 1989; Lord and Anthony 2002; Schroeder et al. 2004; Burkart and Stoner 2007). According to Durfor and Becker (1964), the concentration of nitrate in groundwater may be as much as 1000 mg/l in highly polluted groundwater, but generally, it is found to be less than 50 mg/l. One of the most dominant natural processes that can contribute to reducing nitrate concentrations in groundwater is the process of denitrification. According to Firestone (1982), denitrification most readily occurs above 10 °C and in the presence of electron donors. The process begins with a reduction of NO_3^- to NO_2^-, followed by the reduction of NO_2^- to NO^-, and finally reduction to N_2O or N_2 gases which escapes from groundwater (Rivett et al. 2008).

Investigated groundwater samples that showed low NO_3^- concentrations (<10 mg/l) showed high HCO_3^- (med 272 mg/l), As (med 27.9 μg/l), Fe (med 480 μg/l), and Mg (med 12.18 μg/l) concentrations, pH 7–8, as well as low PO_4^{3-} (med 0.52 mg/l) and SO_4 (med 18.15 mg/l) concentrations suggesting that denitrification due to the reductive environment contributes in decreasing nitrate concentration in this samples. To evaluate this, the chemical properties of groundwater samples were investigated according to Jurgen's calculator in Excel for identifying redox processes in groundwater (Jurgens et al. 2009). The obtained result showed the presence of anoxic conditions in groundwater samples with a low level of nitrate. Considering this it is expected to have low concentrations of SO_4^{2-}, too. Possible natural sources of sulfate in groundwater are dissolution of sulfate-bearing minerals like barite ($BaSO_4$), epsomite ($MgSO_4·7H_2O$), and gypsum ($CaSO_4·2H_2O$), oxidation of pyrite (FS_2), atmospheric fallout, and decomposition of organic matter. Regarding anthropogenic activities, sulfate and sulfuric acid products are used in the production of fertilizers, chemicals, dyes, glass, paper, soaps, textiles, fungicides, insecticides, astringents, and emetics; mining; wood pulp, metal, and plating industries; sewage treatment; and processing of leather (Greenwood and Earnshaw 1984). Fertilizers based on potassium sulfate, ammonium sulfate, and the sulfo-phosphate ammonium are usually applied in the soil. Since sulfate is water-soluble and mobile in the soil, it is expected for soil inputs to have an impact on shallow groundwater. Sulfate exists in groundwater usually as sodium sulfate (Na_2SO_4), calcium sulfate ($CaSO_4$), and magnesium sulfate ($MgSO_4$). In this study investigated groundwater samples with a depth greater than 70 m showed no significant pollution with SO_4^{2-}. The maximum concentration was 166.69 mg/l with a median of less than 5 mg/l. The maximum concentration found in shallow groundwater was 300 mg/l with a median of 21 mg/l. Welch's t-test showed a statistically significant difference between SO_4^{2-} concentrations in deep and shallow groundwater, but the very low level of power analysis (Table 8.4) excludes the possibility to accept this. A positive correlation between SO_4^{2-}, Cl^-, and Na in factor analysis suggests that the majority of sulfate ions is derived in groundwater leaching by rainwater (Table 8.5).

Water percolating through the soil and moving downstream to the aquifer reacts with the surrounding environment composed of rock and minerals that contribute to groundwater characteristic chemical composition. Silicate minerals that comprise most rocks do not react readily with most groundwaters, while carbonate minerals react quite readily with water and thus play an important role in the evolution of many groundwaters. Carbonates are the most common forms of calcium in sedimentary rock formations like calcite and aragonite ($CaCO_3$) and dolomite $CaMg(CO_3)_2$. Other calcium minerals common in sediments include gypsum ($CaSO_4/CaSO_4·2H_2O$) and more rarely fluorite (CaF_2), some types of zeolites, and montmorillonite. Concentrations of calcium in the investigated groundwater range between 7.4 and 411 mg/l with a median value of 39.4 mg/l. No significant difference is observed between shallow and deep groundwater regarding Ca concentration.

The detection frequency of Mn in groundwater in the Strumica region is high which is predicted since it is one of the most abundant metals in earth's crust,

Table 8.5 Factor analysis of major and minor ions in deep groundwater samples of the Strumica region

	F1	F2	F3	F4	F5	Comm
HCO_3^-	0.05	**0.87**	−0.14	0.23	0.04	91.91
Cl^-	**−0.72**	0.06	0.19	0.03	0.14	88.66
NO_3^-	−0.14	−0.18	**0.85**	0.13	0.10	88.78
NO_2^-	0.03	−0.19	**0.64**	−0.13	0.05	64.41
NH_4^+	0.03	−0.01	−0.26	−0.04	**−0.73**	77.22
SO_4^{2-}	**−0.85**	0.01	−0.16	−0.12	0.04	88.52
Na	**−0.68**	002	−0.17	−0.25	0.16	81.42
PO_4^{3-}	0.04	0.11	0.03	−0.02	**−0.83**	77.45
K	**−0.80**	0.02	0.14	0.09	−0.05	92.27
Ca	−0.44	**0.70**	0.19	0.32	0.18	94.42
Mg	0.03	0.45	**0.71**	0.11	−0.30	88.77
As	0.06	**0.70**	0.03	−0.13	−0.05	69.62
Mn	0.07	0.16	0.03	**0.81**	−0.14	80.15
Fe	0.12	−0.08	0.03	−0.07	**−0.65**	53.06
Ni	−0.11	−0.49	0.19	**0.71**	0.12	86.02
Cu	−0.23	−0.32	**0.60**	0.02	0.19	76.54
Zn	0.07	−0.15	−0.14	0.29	0.10	37.36
Mo	−0.01	0.21	−0.20	0.40	0.13	59.60
B	**−0.80**	−0.08	0.14	0.24	0.02	63.68
Pb	0.03	−0.41	0.14	0.03	−0.00	84.36
Al	0.03	0.05	0.27	−0.14	0.02	55.63
Ti	−0.42	0.42	−0.07	**0.60**	0.09	86.72
E-value	3.98	3.05	2.26	1.89	1.63	76.66
Total variance %	18.10	13.86	10.26	8.61	7.41	

usually occurring with iron. Mn can be found as a component of over 100 minerals. In nature, it is not found in its pure (elemental) form (ATSDR 2000). It is a significant constituent of basalt and many olivines, pyroxene, and amphibole. Divalent manganese is a minor constituent in many igneous and metamorphic rocks. Small amounts of divalent magnesium may substitute calcium in dolomite and limestone. In groundwater and other natural waters, Mn^{2+} is a predominant oxidation state and is more stable toward oxidation than the ferrous iron. Mn^{3+} and Mn^{4+} are not likely to occur. Mn^{3+} is unstable except in strongly acidic solutions, and Mn^{4+} could theoretically occur only at very high pH. In aquatic systems where HCO_3^- is present in high concentrations, $MnHCO_3^+$ will be the predominant form (Hem 1963). Manganese concentrations in the investigated groundwater ranged between less than 0.5 μg/l and 3329 μg/l with a median value of 259.76 μg/l. Welch's t-test and power analysis showed that investigated samples from shallow and deep groundwater significantly defer regarding Mn concentrations with median values of 20.8 μg/l and 371 μg/l, respectively. Approximately 45% of shallow and 94% of deep groundwater samples show concentrations greater than the levels of public health concern of 0.05 mg/l. The maximum concentration in shallow groundwater was 3.3 mg/l and in deep groundwater was 2.4 mg/l. According to Annex 3 of the Groundwater Directive 2006/118/EC, only five EU member states established a threshold value (TV) for Mn probably because it is relatively nontoxic and essential for humans and animals. Bulgaria, France, and Spain set a TV of 0.5 mg/l, Poland set a TV of 1 mg/l, and Slovak set a TV of 0.027–0.2 mg/l. This naturally occurring element is ubiquitous in the environment and found in soils, sediments, surface water, and groundwater. It can exist in 11 oxidative states from which the most environmentally and biologically important are Mn^{2+}, Mn^{4+}, and Mn^{7+} (US EPA 1994). Manganese in groundwater may originate from weathering of manganese-bearing minerals and rocks, from rainfall percolating through soils, and industry. The concentration and chemical behavior of Mn in groundwater are controlled mainly by the pH and the redox conditions in groundwater. Acidic and reduced groundwater conditions contribute to greater Mn concentrations. Concentrations up to 1300 μg/l in neutral groundwater and 9600 μg/l in acidic groundwater have been reported by ATSDR (2000). In agriculture, manganese compounds are used in fertilizers and fungicides and as livestock feeding supplements (HSDB 2001).

Sodium in groundwater may originate from rainwater, saltwater intrusion, sewage effluent, and naturally from water-rock interactions or simply the long residence time of water in the aquifer. Sodium is more abundant than potassium in igneous rock but less abundant in sedimentary rocks. Sodium in groundwater shows no important reactions of precipitation that may lead to lowering its concentrations in groundwater such is the carbonate precipitations which control the concentrations of Ca. Sodium can be adsorbed by the clay particles but generally much weaker than divalent ions. During the cation exchange processes in groundwater, divalent ions may be extracted and replaced with monovalent such as Na^+. Concentrations of sodium in the investigated groundwater ranged from 1.39 to 35.86 mg/l with a median of 6 mg/l. Literature data for Na concentration in groundwater situated under the irrigated land showed substantially higher values especially in areas near the saltwater.

The negative effect of Na in irrigated water was estimated by sodium adsorption ratio (SAR) (Richards 1954). The obtained result showed very low values (med 0.28 mg/l) indicating that no hazard from sodium is expected regarding soil permeability if investigated groundwater is used for irrigation.

Chlorine can occur in various oxidation states from Cl^- to Cl^{+7}, but the chloride form has major significance in water. Concentrations reported in the literature data ranged from less than 1 mg/l to more than 200,000 mg/l (Feth 1981). Chloride in groundwater may originate from different sources such as rainwater leaching, saltwater intrusion, domestic and industrial waste discharges, municipal effluents, and sedimentary water-rock interactions. Chloride-bearing minerals occur in igneous rocks like feldspathoid sodalite – $Na_8[Cl_2(AlSiO_4)_6]$ – and apatite $[Ca_{10}(PO_4)_6Cl_2]$, but more important sources are associated with sedimentary rocks like shale.

Concentrations up to 70 mg/l are considered safe in groundwater, while concentrations greater than 350 mg/l may cause severe problems in plant growth (As et al. 2007). Chloride concentration in the investigated groundwater ranged between 4.2

and 614 mg/l with a median value of 24.4 mg/l indicating low pollution. Around 10% of investigated samples showed chloride concentrations greater than 70 mg/l found mostly in shallow groundwater. Although Welch's t-test confirms statistically significant differences between chloride concentrations in shallow and deep groundwater, the power analysis was equal to zero, so this result was rejected, and it is assumed that there are no statistically significant differences between chloride concentrations in shallow and deep groundwater.

Magnesium is a common element in groundwater and together with other alkali earth metals is responsible for water hardness. It may end up in groundwater from many different sources like the chemical industry of plastics, fertilizers, cattle feed, wastewater, and water-rock interactions. Numerous minerals contain Mg like dolomite ($CaMg(CO_3)_2$) and magnesite ($MgCO_3$). The only oxidation state significant in water chemistry is Mg^{2+}. The concentration of Mg in investigated groundwater ranged between 1.1 and 96 mg/l with a median of 9.7 mg/l. The investigations of Mg concentrations in groundwater from different areas showed concentrations in the range from 8.5 to 51,500 mg/l (Hem 1985). The investigations of Pandian and Sankar (2007) of groundwater quality in Vaippar River basin, Tamil Nadu, an area underlain by Precambrian rocks like charnockites, hornblende-biotite gneisses, granite, and crystalline limestones, as well as alluvial deposits along the river basin, showed Mg concentrations in the range of 21.5–92.8 mg/l in post-monsoon and 35.02–91.4 mg/l in the pre-monsoon period. In this study, Welch's t-test showed statistically significant differences between Mg concentrations in shallow and deep groundwater, but the power of the analysis was zero, so this outcome is not accepted, and it is assumed that there is no difference between shallow and deep groundwater regarding Mg concentrations in the investigated region.

Although Mg is an essential element, higher concentrations in irrigated water may be harmful to the plants. The harmful effect of Mg in irrigated water is estimated by the magnesium hazard indices (MH) (Szabolcs and Darab 1964). Water having values greater than 50 is considered unsuitable for irrigation. Around 7% of investigated groundwater samples are unsuitable for irrigation mostly located in the area of the village Dobreici in the northwest part of the Strumica valley.

The orthophosphate ion is more luckily to be present in groundwater than other phosphorous ions. Phosphate can be derived in groundwater from different sources like fertilizers, domestic and industrial sewage effluents, and detergents, but since phosphates are not very mobile in soils or sediment, its presence in groundwater is prescribed mostly to the water interactions with phosphate-bearing minerals like apatite and brushite ($CaHPO_4{\cdot}2H_2O$). Concentrations in investigated groundwater were low and range between less than 0.01 and 7.8 mg/l with a median of 0.18 mg/l.

The most common form of iron in groundwater is the ferrous ion Fe^{2+}. Iron concentrations of 1–10 mg/l in groundwater are common for many areas. Sources of iron in groundwater are fertilizers, industry, and mineralization. Igneous rock minerals high in iron content include the pyroxenes, amphiboles, biotite, magnetite, and, especially, the nesosilicate olivine. When these minerals react with groundwater, the released iron is reprecipitated. In reducing conditions and high sulfur content, ferrous polysulfides may be formed. If the sulfur is less abundant, then the siderite ($FeCO_3$) is most likely to occur. The activities in the biosphere may also influence iron concentrations in groundwater since microorganisms are commonly involved in iron oxidation and reduction processes. Investigated groundwater have iron concentrations in the range from less than 0.05–3166 μg/l with a median of 69.8 μg/l. Statistically, significant differences are observed between Fe concentrations in shallow and deep groundwater (Table 8.4) with median values of 57.8 μg/l and 161 μg/l, respectively.

Potassium in groundwater may originate from agriculture activities mainly fertilizer application, rainwater, and organic matter in soil, but the considerable amount may be released from igneous and metamorphic rocks like silicate minerals, orthoclase, microcline, hornblende, muscovite, and biotite. It is less common in igneous rock than sodium but more common in all the sedimentary rocks. It is assumed that potassium concentrations in groundwater are low because of the high degree of stability of potassium-bearing aluminosilicate minerals. Generally, the potassium ion is adsorbed less strong than sodium ion in ion exchange reactions mainly because it is larger, but in illite, it is incorporated in the free space between the crystal layers and thus difficult to be removed by future ion exchange interactions. Concentrations of potassium in investigated groundwater ranged between 0.7 and 354 μg/l with a low median value of 5.4 μg/l. No significant differences were observed between potassium concentrations in shallow and deep groundwater.

Piper diagram was used to identify major hydrogeochemical processes in the groundwater of the investigated region. For that purpose, the concentrations of major cations like Ca, Mg, Na, and K and major anions like CO_3^{2-}, HCO_3^-, Cl^-, and SO_4^{2-} were expressed in meq/l and normalized to the percentage of the individual cation/anion concentration relative to the total cation/anion concentration, respectively. Piper diagram was plotted using the statistical program Grapher 16.2.354 (2020). The result showed that 91.30% of the total 207 investigated samples belong to the $CaHCO_3$ water type suggesting that carbonate rock watering plays a significant role in the groundwater chemistry of the Strumica region. The remaining 8.7% of the investigated samples belong to the Ca-Mg-Cl and Na-HCO_3 type of water (Fig. 8.2). Such hydrological characteristics of the groundwater are reflected from the geological formations of the investigated area. Carbonates are present in many different types of rock. Quaternary alluvial and Neogene clastitic sediments, Paleozoic shales, Proterozoic gneisses, and magmatic rock are common for the region of Strumica (Čančalova et al. 2017). Since calcium is a major constituent of many common rock minerals and the most abundant alkaline-earth metal, it appears as a major component of the solutes in most natural water.

8.3.1.2 Minor Components

Minor constituents in groundwater generally occur in concentrations lower than 1 mg/l. From the descriptive statistical analysis of the investigated minor constituents given in Table 8.3, it can be seen that minor components also occur in a wide range of concentrations indicating that groundwater in the investigated region is

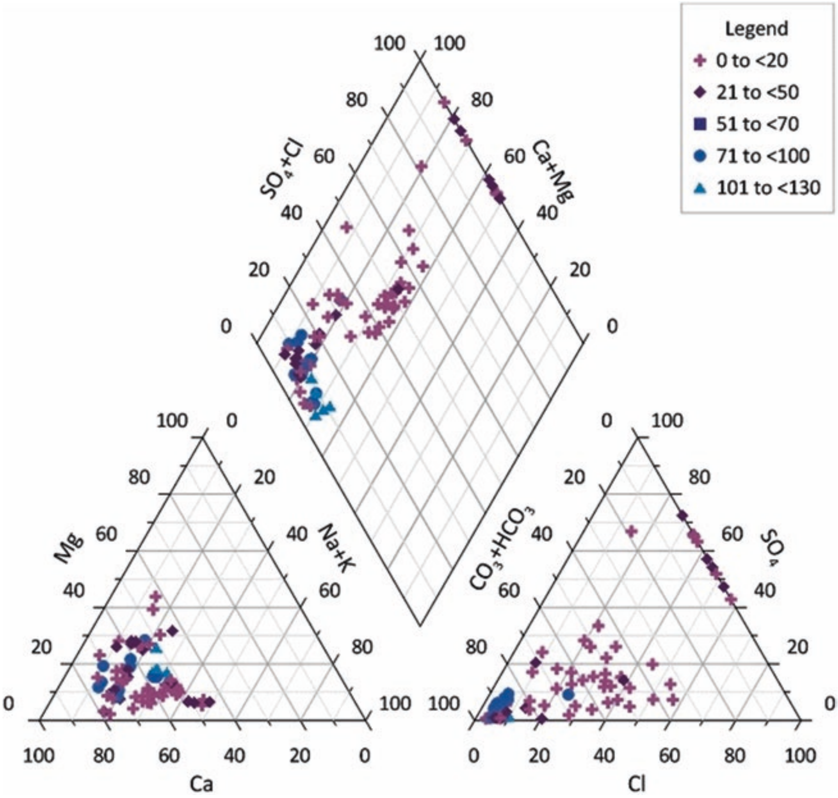

Fig. 8.2 Piper diagram for groundwater type of the Strumica region

not uniform in its quality regarding minor component concentrations. Welch's t-test was performed for normal and Box-Cox transformed values showing significant differences between shallow and deep groundwater regarding As, Ba, Mo, Pb, and Ti concentrations when normal values are processed and between As, Ba, Mo, Ti, and Zn when Box-Cox transformed values are processed (Table 8.4). The power analysis showed a high percentage of accuracy (>95%) only for As and Ba when normal values are processed and for As, Ba, Ti, and Zn when Box-Cox normalized values are processed. The median values for these elements and ions are higher in deep groundwater except for Ti which was found that have significantly greater concentrations in shallow groundwater. Median values for Ti in shallow and deep groundwater were 152 μg/l and 131 μg/l with a maximum concentration of 1036 μg/l. The average content of Ti in igneous rock is around 4.83 g/kg, while in sedimentary rock, the content is 4.44 g/kg, 1.95 g/kg, and 0.38 g/kg for shale, sandstone, and carbonates (Chilton 1996). The important anthropogenic source of Ti is the aircraft and space industry. The oxide is used principally as a white pigment in paint. Such industries are not common for the investigated region, so it is assumed that the presence of Ti in groundwater has a lithological origin. The element is common and widely distributed in rocks and soil, and thus it is naturally present in groundwater (Hem 1985).

Arsenic is highly undesirable in groundwater. Stable forms in water solution are arsenate (As^{5+}) and arsenite (As^{3+}) oxyanions. It may exist in the form of sulfides in the sulfide ore deposits. Considering the mean values, higher concentrations of As are present in deep groundwater with a maximum measured concentration of 177 μg/l (Table 8.3). Elevated concentrations of arsenic are frequently found in groundwater worldwide (Ravenscroft et al. 2009), and the pollution of groundwater with this toxic element has become a global concern problem. Most of the EU member states have established a threshold value (TV) of 10 μg/l for groundwater bodies. Polluted groundwater has been found in many parts of the world in different hydrogeological and geochemical conditions. Literature data show that majority of the arsenic polluted groundwater is mostly affected by natural contamination when As is released from the natural bedrock of the aquifer (Rosas et al. 1999; Smedley and Kinniburgh 2002; Smedley et al. 2005; Berg et al. 2001; Polya et al. 2005). Other sources like mining activities, industry, and the use of arsenic pesticides are also found as contributors for elevated As concentrations in groundwater (Twarakavi and Kaluarachchi 2006; Bernard 1983; Grantham and Jones 1977; Smedeley et al. 2007; Komnitsas et al. 1995; Chakraborti et al. 1999; Pandey et al. 2002; Bednar et al. 2002; Cai et al. 2002; Wiegand 1999). Arsenic-bearing rocks like calcite forms of limestone, iron oxide minerals, and sodium feldspars are common for the investigated region (Rakicevik and Pendzerkoski 1973). In the Strumica region, almost 35% of investigated groundwater samples showed As concentration greater than 10 μg/l from which 16% have concentration greater than 50 μg/l. There are four mobilization mechanisms of As in nature: reductive dissolution, alkali desorption, sulfide oxidation, and geothermal (Ravenscroft et al. 2009). Reducing environment present in the groundwater of the investigated area; high Fe, Mg, and HCO_3; and low SO_4 and NO_3 content suggest that reductive dissolution is a major mechanism by which arsenic is released into the groundwater. Arsenic-polluted samples from the investigated region containing more than 50 mg/l of arsenic are characterized by low concentrations of SO_4 (med 7.38 mg/l), PO_4 (med 1.08 mg/l), K (med 5.03 mg/l), Ca (med 42.58 mg/l), Mg (med 9.63 mg/l), Cu (med 0.85 mg/l), Zn (med 21.57 mg/l), and Pb (med 1.13 mg/l).

Boron concentration in investigated samples ranged from less than 0.5 to 136 μg/l with a low median value of 6.25 μg/l. It is an essential element for plants but in higher concentrations may be harmful. Therefore, boron concentrations in groundwater are very important if it is used for irrigation purposes. The most sensitive to boron are lemon and orange trees and blackberry. The lowest toxic concentrations cited in the literature data are 0.75 mg/l. Such concentrations were not observed in the investigated groundwater.

The presence of barium in groundwater can suggest the presence of industrial waste pollution, saltwater intrusion, mixing of natural saline and brine waters, and dissolution of Ba-bearing minerals like barite ($BaSO_4$). Barium concentrations in investigated groundwater ranged between 1.25 and 128 μg/l with a median value of

29.6 μg/l. Statistically significant differences were observed between shallow and deep groundwater in the investigated region regarding Ba concentrations (Table 8.4).

Sources of Zn in groundwater include textile industries and battery production, agricultural fertilizers and sprays, and metallurgical activities like galvanizing of metal and alloy production. It is present in minerals like shale (95 mg/kg), sandstone (16 mg/kg), limestone (20 mg/kg), and igneous rock (70 mg/kg) (Hem 1972). The concentrations in the investigated region ranged between 2.34 and 1371.41 μg/l with the mean value of 13.97 μg/l. Zinc was found in significantly higher concentrations in deep groundwater with a median value of 16.03 μg/l compared to the shallow groundwater with a median of 11.50 μg/l.

8.3.2 Factor Analysis

Factor analysis applied for all investigated samples to identify the association between investigated variables was less than satisfactory since obtained factors account for only 36.82% of the total variance and 45.64% of communalities. It is assumed that this outcome arises from an inadequate data processing approach. Groundwater in the investigated region is constantly pumped and circulates through the soil. Such practice contributes to the accumulation of certain elements in the upper soil layers and their leaching downstream to the aquifer. Shallow groundwater situated under irrigated land will be more affected with the pollution of these elements which may result in unreliable results from the conducted statistical analysis if shallow and deep groundwaters under the irrigated land are statistically processed together. Therefore, another factor analysis was performed processing only deep groundwater samples. The loading values for each variable, E-values, and communalities are given in Table 8.5. The analysis revealed five factors that account for almost 60% of the total variance and total communalities of 76.66%. The factor associations in deep groundwater are F1 (Cl, SO_4, Na, K, and B), F2 (HCO_3^-, Ca, As, Mg), F3 (NO_3^-, NO_2^-, Mg, Cu), F4 (Mn, Ni, Ti), and F5 (NH_4^+, PO_4, Fe). The first factor (Cl^-, SO_4^{2-}, Na, K, and B) associates the most affected ions by rainwater leaching through the soil. Extensive studies on rainfall composition showed that Cl, SO_4, and Na are the most abundant components in rainwater (Hem 1985). The first factor positively correlates these ions suggesting that the origin of these ions is probably associated with the leaching of rainwater. Potassium is an essential element for plants. It is considered that its content in plants is near 0.3% (Mason 1952) meaning that its concentrations in dry plant material will be higher so the organic residues in soil may present a considerable source of potassium that may be affected by rainwater leaching. The second factor F2 (HCO_3^-, Ca, As, and Mg) makes association between ions that arise from the water-rock interactions and main hydrogeochemical reactions that arise from groundwater hydrology. As it was previously mentioned, investigated groundwater belongs to the Ca-HCO_3 type, and carbonate rock watering plays a significant role in groundwater chemistry. In the presence of high HCO_3^- ions, reducing conditions, and almost neutral pH, the reductive dissolution of Mg oxides will mobilize As in water (Quevauviller et al. 2009). The third factor F3 (NO_3^-, NO_2^-, Mg, and Cu) positively correlates NO_3, NO_2, Mg, and Cu and associates ions that are affected by the reduction processes in groundwater. The first step of the denitrification process is the reduction of NO_3^- to NO_2^-. The presence of Mg and Cu in this factor and the positive correlation with NO_3^- and NO_2^- indicate to the reduction of these elements in groundwater. The fifth factor F5 (NH_4^+, PO_4^{3-}, and Fe) associates ions which are related to the use of fertilizers.

8.4 Conclusion

The quality of groundwater situated under agriculture land was investigated in this study regarding major and minor components, some heavy metals, and trace elements. The descriptive analysis showed that groundwater in the Strumica region is not uniform but differs considerably in major and minor component concentrations. Groundwater samples were considered in terms of their depth and processed as shallow (<70 m) and deep (>70 m) groundwater. Higher median values were observed for HCO_3^-, NO_3^-, SO_4^{2-}, Cl^-, Ca, Mg, and K concentrations in shallow groundwater, while Fe, Mn, Na, PO_4^{3-}, and NH_4^+ showed greater concentrations in deep groundwater. Welch's t-test followed by power analysis was used to investigate differences between cations and anions in shallow and deep groundwater. The result indicates significant differences in NH_4^+, PO_4^{3-}, Fe, Mn, As, Ba, Ti, and Zn concentrations. Piper diagram plotted to identify groundwater chemistry showed that the majority of groundwater falls under the rock dominance category and belongs to the Ca-HCO_3 water type suggesting that carbonate rock watering plays a significant role in the groundwater chemistry of the Strumica region. High HCO_3^- (med 272 mg/l), As (med 28 μg/l), Fe (med 480 μg/l), and Mg (med 12.2 μg/l) concentrations, as well as low PO_4^{3-} (med 0.52 mg/l) and SO_4^{2-} (med 18.2 mg/l) concentrations and pH 7–8 in samples with low nitrate concentrations (<10 mg/l), suggest the presence of reductive environment which contribute in the process of denitrification at almost 65% of investigated groundwater samples. Almost 35% of investigated groundwater samples showed As concentration greater than 10 μg/l from which 16% have concentration greater than 50 μg/l with a maximum concentration of 177 μg/l. Reducing environment present in the groundwater of the Strumica region; high Fe, Mg, and HCO_3^-; and low SO_4^{2-} and NO_3^- content suggest that reductive dissolution is a major mechanism by which arsenic is released into the groundwater. Factor analysis performed to access of investigated major and minor components in groundwater of the Strumica region identify four significant factors. The amount of Cl^-, SO_4^{2-}, Na, K, and B in groundwater is attributed to the rainwater leaching through the soil. The amount of HCO_3^-, Ca, As, and Mg is attributed to the main hydrogeochemical reactions that exist in groundwater of the investigated region. The amount of NO_3^-, NO_2^-, Mg, and Cu are related to the reduction processes that influence groundwater quality, and the amount of NH_4^+, PO_4^{3-}, and Fe is attributed mostly to the use of fertilizers in the investigated region. When groundwater quality situated under the agriculture activities is the purpose of investigation and its main use is for irrigation, it is suggested to perform factor analysis for deep and shallow groundwater samples separately if statistical analysis shows significant differences between shallow and deep groundwater, to achieve more reliable results. Constant pumping of groundwater and its permanent circulation through the soil layers will tend to influence the soil quality and have a different effect on shallow and deep groundwater. These differences may lead to confusing results when variables from shallow and deep groundwater are statistically processed together.

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Chapter 9
Removal of Chromium(VI) from Aqueous Solution by Clayey Diatomite: Kinetic and Equilibrium Study

Hamdije Memedi, Katerina Atkovska, Stefan Kuvendziev, Mrinmoy Garai, Mirko Marinkovski, Dejan Dimitrovski, Blagoj Pavlovski, Arianit A. Reka, and Kiril Lisichkov

Abstract The clayey diatomite from the village of Suvodol (Bitola region) used in this research has shown to be a very effective and efficient natural material for the removal of Cr(VI) ions from aqueous solutions. The adsorption of Cr(VI) ions onto a clayey diatomite was shown to occur very efficiently with 96.7%. During the experiments the effect of operating parameters, such as amount of adsorbent (0.5–5.5 g/l), initial adsorbate concentration (0.3, 0.4, 0.5, and 0.6 mg/l), and time of adsorption (5–180 min) at pH of the solution 3, was examined. It was found that 2.5 g/l is optimal adsorbent dosage for maximal removal of Cr(VI) ions. Atomic absorption spectrophotometric (AAC) method and UV-Vis spectrophotometric analysis are used for quantitative monitoring of the dynamics of the studied system regarding the presence of Cr(VI) ions in the model solutions. The effect of the initial concentration and quantity of adsorbent is examined, in order to determine the conditions that enable maximum removal of Cr(VI) ions with clayey diatomite. For the purpose of modeling the equilibrium, the kinetics, and dynamics of the tested system of the process of elimination of Cr(VI) ions with the raw material in different operating conditions, MATLAB/Curve Fitting Toolbox was conducted.

Keywords Adsorption · Equilibrium · Kinetics · Clayey diatomite · Cr(VI) ions

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9.1 Introduction

Metals are present in all the parts of our environment. Some of the metals at certain levels are important for maintaining life functions, while some metals can be harmful to the human body. The presence of heavy metals in water resources can be of geogenic or anthropogenic origin. The industrial production takes part with the largest percent emission of heavy metals, which are of anthropogenic origin. Many of the metals are toxic even in very low concentrations, due to their potential to accumulate in the living organisms and thus enter the food chain. Heavy metals released into the aquatic environment are mostly tied to deferred materials, and they eventually accumulate in sediments (Tchobanoglous et al. 2003; Grady Jr et al. 2011).

Among the various metals such as heavy metals that occur as most toxic pollutant of wastewater, chromium is ranked as one with the highest toxicity. The toxic effect of chromium is correlated with its valence. The six-valent chromium Cr(VI) is toxic to all life forms and is considered the main pollutant (Norseth 1981; Toxicological Profile for Chromium 1991; Cieslak-Golonka 1995; Myers et al. 2000; Zhitkovich et al. 2002; Dupont and Guillon 2003). Cr(VI) is used in many industries such as the manufacturing of paints, metalworks, manufacture of automobile parts, and in the petrochemical industry (Chakir et al. 2002). Wastewater from these industries contains significant quantities of chromium that is spread in the environment through land and water and eventually accumulates through the food chain. It is soluble in water and can adsorb in living organisms. When it accumulates in living organisms, it can cause major health problems. Exposure to lower concentrations may cause lung tumor and liver, kidney, and other reproductive problems. The International Agency for Research on Cancer has classified chromium as a carcinogen (Biswajit et al. 2013). Hexavalent form of chromium, Cr(VI) is the most damaging for the health of humans due to its carcinogen and mutagenic properties. Therefore, there are legal regulations governing the permitted amounts of heavy metals that are found in water resources, depending on their further use. Of particular importance are defined MAC values (maximum permissible concentrations) for each element separately, which are the result of extensive consultation of the scientific research literature and represent the prescribed standards. Although the limit for Cr(VI) in the wastewater is only 0.05 mg/L, the actual found amounts are much higher (Case Studies in Environmental Medicine: Chromium toxicity 2000).

In recent years, adsorption proves to be an alternative method for the removal of metal ions from the wastewaters, including the Cr(VI). To reduce capital costs, there have been major studies on the use of cheaper adsorbents which may be different types of soils and clays. Removal of heavy metals with the use of soils is performed based on the great adsorption capacity of the negatively charged parts of the soil and the possibility of formation of complex molecules with organic molecules that are located in the soil and thus resulting in the formation of oxides, hydroxides, and other insoluble minerals. Clays show such properties, as a result of their chemical and mechanical stability, large surface area, permanent negative voltage, and other surface and structural properties (Babel and Opiso 2007). For this purpose, various raw natural materials such as red mud (Gupta et al. 2001a), silicates (Chiron et al. 2003; Zendelska et al. 2018a), glauconite (Belousov et al. 2019), and natural (Zendelska et al. 2015a, b, 2018b, 2019) and modified zeolites (Wingenfelder et al. 2005) are used. The removal of chromium, Cr(VI), is tested with adsorbents such as activated carbon (Srivastava et al. 1996), *Spirogyra* biosorbents (Gupta et al. 2001b), and fly ash (Bayat 2002; Gupta and Ali 2004). The soybean, tires, shells from hazelnuts, and sawdust have also been used for the same purpose. In recent years, the elimination of heavy metals from wastewater is achieved by the use of certain types of natural or agricultural wastes (Chatterjee et al. 2010; Khambhaty et al. 2009), containing various functional groups such as hydroxyl, aldehyde, aliphatic acid group, alkene, amide, nitroaromatics, silicate, sulfonate, and others. It is the existence of these functional groups in the organic adsorbents that enables the adsorption of Cr(VI). Also for this purpose, in recent years, the effectiveness of removal of the six-valent chromium using natural low-cost adsorbents, such as sunflower stalks (Sun and Shi 1998), crust of *Eucalyptus* (Sarin and Pant 2006), bran of corn (Singh et al. 2006), coconut shell, leaves of trees, shells of peanuts, and nuts (Karthikeyan et al. 2005), has been explored. Chromium six-valent as well as other pollutants can also be removed with inorganic raw materials such as bentonite, aksil, and pemza (pumice) (Memedi et al. 2016a, b, 2017; Belousov and Krupskaya 2019).

Republic of North Macedonia is rich in natural inorganic materials such as clayey diatomite (Pavlovski et al. 2011; Cekova et al. 2013; Reka et al. 2016, 2019b), perlites (Reka et al. 2019a), and diatomite (Reka et al. 2014, 2017) that represent industrial minerals which could be used to successfully remove various metal ions from aqueous solutions.

9.2 Materials and Methods

The inorganic raw material used as adsorbent of Cr(VI) during this research is collected from the village of Suvodol, Bitola, Republic of North Macedonia. During this research clayey diatomite is used in its natural state, without any modification or activations.

The chemical composition of the material was determined with the classical silicate analysis, while the XRPD analysis was performed on the DRON diffractometer, 36 kV, 18 mA, CuKα/Ni. The infrared analyses were performed on PerkinElmer FTIR 2000, by KBr tablet technique. The scanning electron microscopy (SEM) of clayey diatomite was performed with energy dispersive X-ray spectroscopy (EDX) employing FEI Quanta 3D FEG dual beam microscope, and the transmission electron microscopy was performed with Hitachi H-7650 apparatus (120 kV automatic microscope).

For the preparation of solutions of Cr(VI) with a particular initial concentration, $K_2Cr_2O_7$ standard solution with a concentration of 1000 mg/l was used. pH of solutions was adjusted using 0.1M HCl or 0.1M HNO_3 and 0.1M NaOH. All reagents and chemicals used in the experiments are of analytical grade.

The quantitative monitoring of the dynamics of studied system regarding the presence of Cr(VI) ions in the model solutions was performed with the atomic absorption spectrophotometric (AAS) method using AAC PerkinElmer model AA700 and UV-Vis spectrophotometric analysis instrument type HACH DR/2010.

In order to determine the mass loss and the characteristic points of possible physical and/or chemical changes occurring in the materials tested in the temperature range which is examined, thermogravimetric and differential thermal analyses (TGA/DTA) were performed. Test apparatus used during this analysis was PerkinElmer Pyris Diamond thermogravimetric/differential thermal analyzer, in air conditions, at rate of heating of 10 °C/min, using 15 mg of sample.

The specific surface area was conducted by the BET method for N_2 adsorption. Determination of the point of zero charge of clayey diatomite was conducted by adding 0.2 g diatomite in Erlenmeyer flasks (300 ml) and adding 100 ml of distilled water with pH ($pH_{initial}$) from 2 to 10. pH is adjusted by adding solution of HCl or NaOH as required. Erlenmeyer flasks were continuously mixed on stirrer (140 rpm) for 24 hours at room temperature. The suspension was then filtered and the pH (pH_{final}) of every filtrate was determined. The pH of the solutions was measured by pH meter GMH 3500 series, Greisinger, Germany.

9.2.1 Adsorption Experiment

In order to define the adsorption characteristics or adsorption affinity and capacity, the raw material was tested in the direction of separating of Cr(VI) ions from model aqueous solutions, as well as monitoring the dynamics of the studied process; a series of batch adsorption experiments were performed. These experiments were conducted in the laboratory glass reactor with a working volume of 2 l, equipped with a magnetic stirrer at room temperature and constant speed of stirring at 400 rpm, while monitoring the equilibrium and the kinetics of the process. During the experiments, the influence of the following operating parameters on the process of removal of Cr(VI) ions was studied: initial concentration of adsorbate (0.3, 0.4, 0.5, and 0.6 mg/l), time of adsorption (5–180 min), and the amount of adsorbent (0.5–5.5 g/l). The pH of the solutions was 3. For the preparation of standard solutions with the desired initial metal concentration, a standard solution of $K_2Cr_2O_7$ was used.

The values of initial concentrations of the working adsorbent are selected in a given operating range based on the MPL statutory regulation values for drinkable and wastewaters with heavy metals multiple times over the allowed limit. Thus, this covers the areas of potential pollutants of water resources that would originate from geogenic or anthropogenic origin. The results of the experiments are used to modulate the equilibrium of the adsorption process through the implementation of several adsorption isotherms (Langmuir, Freundlich, Langmuir-Freundlich, and

Redlich-Peterson), using the software package MATLAB/Curve Fitting Toolbox. In order to define the kinetics of the studied systems, the following kinetic models were applied: pseudo-first- and pseudo-second-order models and model of Elovic.

9.3 Results and Discussion

9.3.1 Physical-Mechanical Characterization of Clayey Diatomite

Based on the macroscopic examinations, the clayey diatomite represents a fairly soft, loose, weakly bound sedimentary rock with gray color. The subject diatomite is of biogenic origin, with compressive strength in natural state 3.30–3.40 MPa (Fig. 9.1).

The sample is characterized by low bulk density of less than 1 g/cm^3 and an appropriately high porosity. The specific surface area (a_s, m^2/g) is determined by BET method and is 18.03 m^2/g. In Tables 9.1 and 9.2 are shown the physical and mechanical characteristics of clayey diatomite and the chemical composition.

9.3.2 X-Ray Analysis (XRD)

The results of the X-ray powder diffraction of clayey diatomite are shown in Fig. 9.2. Based on the results, it is concluded that clayey diatomite is consisted of the following minerals: quartz (peaks at 20.87°, 26.67°, 39.50°, 50.15°, 59.75°), plagioclase (peaks at 13.88°, 18.84°, 23.58°, 24.32°, 24.66°, 27.82°, 27.95°, 30.24°, 30.52°),

Fig. 9.1 A piece of clayey diatomite in natural state

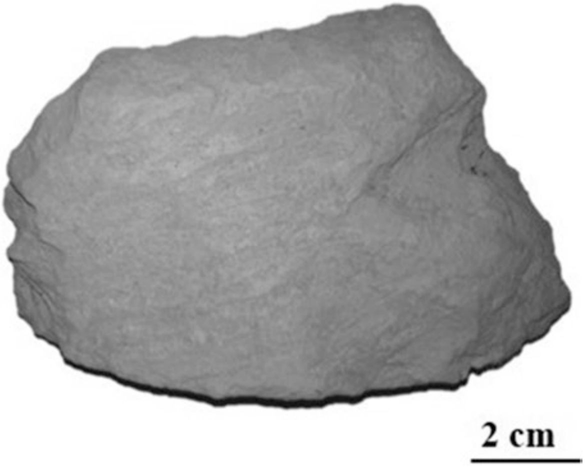

Table 9.1 Physical-mechanical properties of clayey diatomite

Property	Amount
Density (g/cm^3)	2.45
Bulk density (g/cm^3)	0.68–0.79
Water absorption (%)	70–78
Open porosity (%)	53–62
Closed porosity (%)	65–72

Table 9.2 Chemical composition of clayey diatomite

Oxide	SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	K_2O	Na_2O	SO_3	LOI	Total
Content, %	64.95	11.85	4.51	1.49	1.88	1.40	0.84	1.74	11.20	99.86

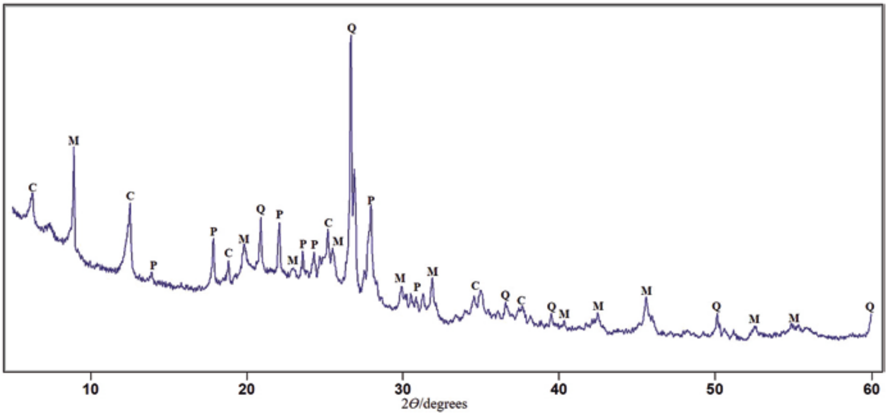

Fig. 9.2 X-ray examinations of clayey diatomite (Q quartz, M muscovite, C chlorite, P plagioclase)

muscovite (peaks at 8.91°, 17.85°, 19.79°, 26.92°, 29.87°, 31.85°, 34.51°, 38.19°, 42.15°, 45.59°, 45.94°, 52.54°, 55.53°), and chlorites (peaks at 6.26°, 12.54°, 18.85°, 25.21°, 25.53°, 27.82°, 31.28°, 37.41°, 37.65°, 39.50°, 45.59°, 45.94°). The bulge in the background in the region 20–27 2θ is due to the presence of the amorphous SiO_2 (short range order of silicate Si-O-Si) in clayey diatomite.

9.3.3 FTIR (Fourier Transform Infrared) Analysis of Clayey Diatomite

The IR spectrum of the clayey diatomite (Fig. 9.3) exhibits an absorption band at 796 cm^{-1} attributed to the bending vibrations of Si-O-Si framework, while the band at 1035 cm^{-1} is a result of the stretching vibrations of Si-O-Al units (Garai et al.

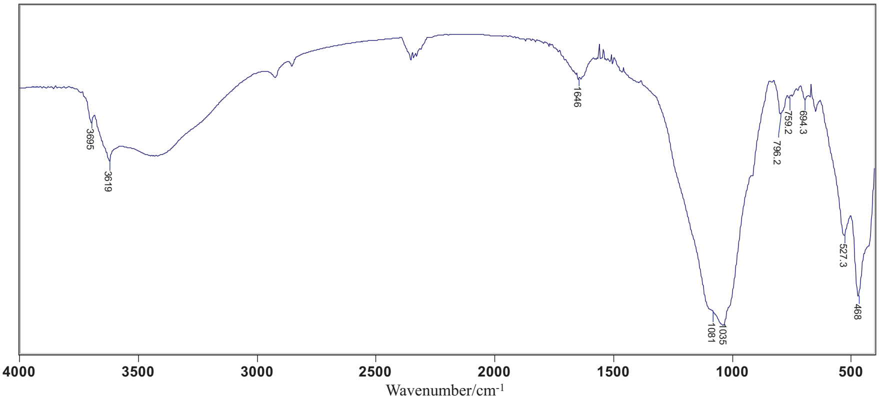

Fig. 9.3 FTIR spectra of raw clayey diatomite

2014, 2015a, 2020). The band at 1646 cm^{-1} is due to bending vibrations from the absorbed water, while the wide band around 3400 cm^{-1} is due to the stretching vibration of the absorbed water molecules (Garai et al. 2015b). The bands at 468 cm^{-1}, 527 cm^{-1}, and 694 cm^{-1} are due to the presence of feldspars in the sample (Chukanov 2014). The bands at 3695 cm^{-1}, 3619 cm^{-1}, and 759 cm^{-1} are due to the clay minerals present in the sample (Chukanov 2014). The bands at 796 cm^{-1} and the shoulder at 1081 cm^{-1} are due to the presence of quartz in the sample (Krupskaya et al. 2019).

9.3.4 Thermal and Thermogravimetric Analysis

Based on the TGA curve of the clayey diatomite, it is evident that there is a mass loss during the heating process. The thermogravimetric analysis (TGA) of the analyzed clayey diatomite shows weight loss in three temperature intervals (Fig. 9.4). The first temperature interval is between room temperature and 100 °C. The mass loss in this temperature interval is 2.9% and is attributed to the free water found in the sample. The second temperature interval occurs between 100 °C and 590 °C, where the mass loss is 9.45%. This mass loss is attributed to dehydroxylation of the clay component (muscovite and chlorite). The third temperature interval occurs at temperatures higher above 590 °C. The thermogravimetric curves continually show lower intensity of mass loss (around 1%) as results to the dehydration process of the opal component in the clayey diatomite.

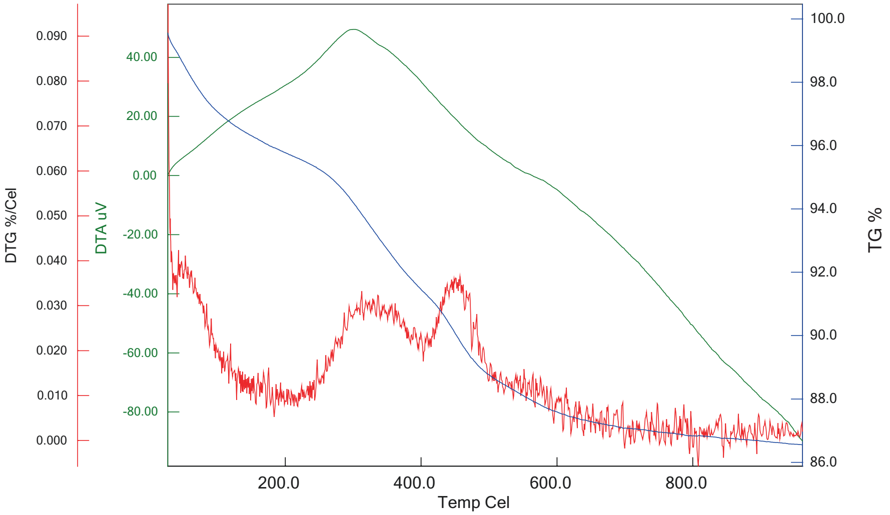

Fig. 9.4 DTA/TGA of clayey diatomite

9.3.5 SEM (Scanning Electron Microscopy) Analysis

Scanning electron microscopy is performed in order to obtain important information regarding the biogenetic origin of the raw material and its morphological characteristics. The results of the scanning electron microscopy of the clayey diatomite are shown in Fig. 9.5. The fragments from the different skeletal forms of microfossils are clearly visible as well as the large number of pores present in the raw material. The sizes of the pores found in the clayey diatomite are in the range 310–650 nm.

9.3.6 TEM Investigations

Transmission electron microscopy results (similarly to SEM) show the presence of the various microrelics and their morphology. TEM images show globular structures from opal nature with biogenetic origin and relics with different elongated shapes and other shapes which are most likely due to the clay minerals present in the sample (Fig. 9.6).

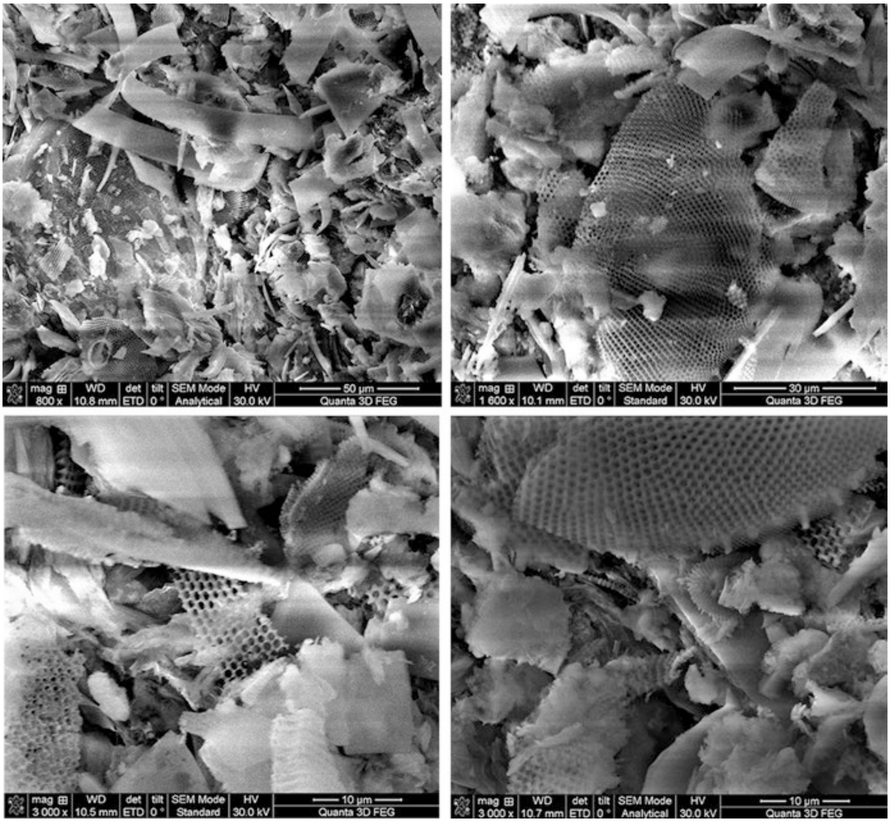

Fig. 9.5 SEM photographs of clayey diatomite showing various microfossils and their skeletal forms with clearly visible pores

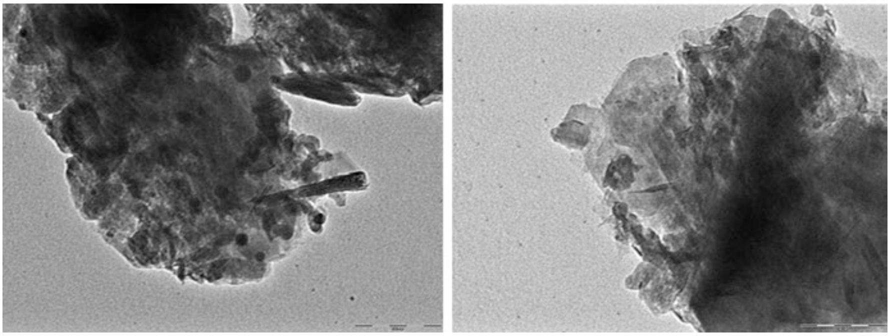

Fig. 9.6 TEM images of clayey diatomite

9.3.7 Adsorption Studies

The effects of amount of adsorbent, initial metal ion concentration, and the time of adsorption were investigated. The percentage of removal of Cr(VI) ions is determined using Equation 9.1.

$$\%R = \frac{\gamma_0 - \gamma_e}{\gamma_0} \cdot 100 \tag{9.1}$$

where γ_0 is the initial concentration of Cr(VI) ions in solution [mg/l] and γ_e is equilibrium concentration [mg/l].

The adsorption capacity at time t, q_t [mg/g], is determined using the Equation 9.2.

$$q_t = \frac{(\gamma_0 - \gamma_t)V}{m} \tag{9.2}$$

where γ_t [mg/l] is the concentration of the metal at time t, V is the volume of the solution [l], and m is adsorbent mass [g].

9.3.7.1 Amount of Adsorbent

Examining the impact of the amount of adsorbent on the process of adsorption is a very important parameter that determines the potential of the adsorbent for removal of metal ions in particular initial concentrations. The influence of the amount of clayey diatomite (0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 g/l) in the degree of removal of Cr(VI) ions was studied for the constant initial metal concentration of 0.5 mg/l, at room temperature, pH 3, at 400 rpm. Results show that by increasing the amount of adsorbent, the percentage of removal of Cr(VI) increases as well. Increasing the amount of adsorbent provides greater contact area and the availability of multiple active zones, leading to improve adsorption of test ions (Chatterjee et al. 2010). In adsorbent amount of more than 2.5 g/l, the removal of Cr(VI) is reduced due to the agglomeration of particles of clayey diatomite and thus resulting in the decrease of active surface of the diatomite (Fig. 9.7).

9.3.7.2 Initial Metal Ion Concentration

The experiments for investigation of the influence of the initial metal ions concentration on the percentage of removal were performed with a constant amount of clayey diatomite of 2.5 g/l at pH of the solution 3, at constant room temperature, and different initial Cr(VI) ion concentrations of 0.3, 0.4, 0.5, and 0.6 mg/l. The impact of the initial concentration in the percentage of removal of diatomite is shown in Fig. 9.8. The figure shows that the rate of removal increases by decreasing of the initial concentration of Cr(VI) ions. The lowest initial concentration of 0.3 mg/l has

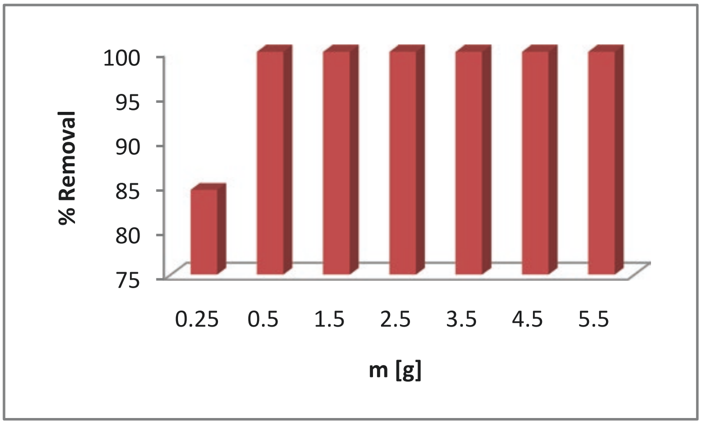

Fig. 9.7 Effect of amount of adsorbent to the rate of removal of Cr(VI) ions

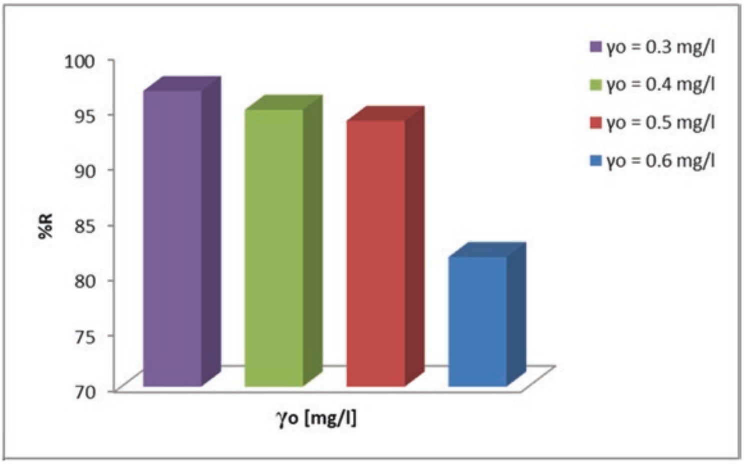

Fig. 9.8 Effect of initial concentration on the adsorption process of Cr(VI) ions in clayey diatomite

the percentage of removal of 96.7%, and then it decreases to 95, 94, to 81.7% by increasing the initial concentration of Cr(VI) ions of 0.4, 0.5, and 0.6 mg/l, respectively. At the lower initial metal ion concentration, there are sufficient active centers on the surface of the adsorbent, and therefore the percentage of removal is higher.

9.3.7.3 Time of Adsorption

The experiment was performed over the period of 180 min, at 2 l solutions of Cr(VI) with different initial concentration, using 2.5 g/l clayey diatomite, at pH 3, room temperature, and 400 rpm. The dependence of the concentration and the adsorbed amount

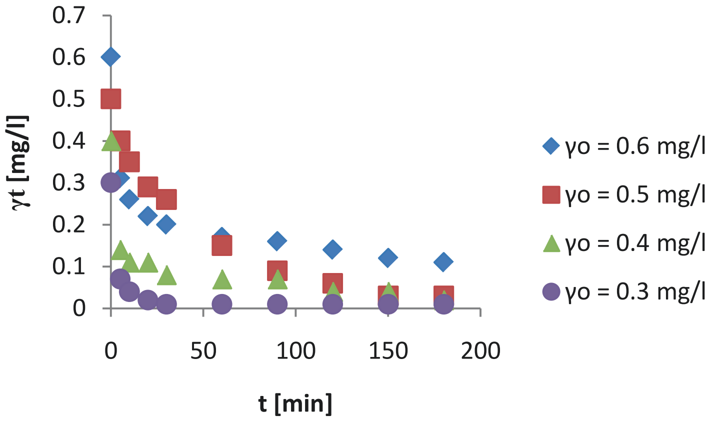

Fig. 9.9 Dependence of adsorbate concentration from time of adsorption for the system Cr(VI) ions – clayey diatomite

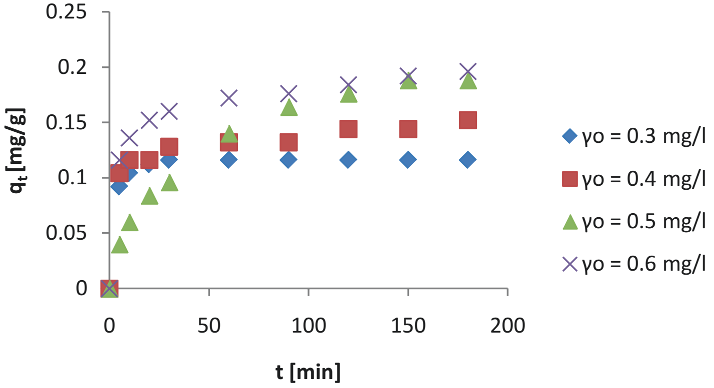

Fig. 9.10 Dependence of adsorbed amount of Cr(VI) from time of adsorption for the system Cr(VI) ions – clayey diatomite

of Cr(VI) ions from the time of adsorption is shown in Figs. 9.9 and 9.10. Analyzing the results, it can be concluded that the tested systems reach the equilibrium after 45–60 min of adsorption for initial concentrations of 0.3 and 0.4 mg/l, while it took 120–150 min to reach the steady state for the concentrations of 0.5 and 0.6 mg/l.

9.3.8 Equilibrium of the System Cr(VI) Ions-Clayey Diatomite

Obtaining experimental data for the system Cr(VI) ions-clayey diatomite is performed using four equilibrium isotherms as follows: Langmuir, Freundlich, Langmuir-Freundlich, and Redlich-Peterson. Selecting the proper isotherm is important for determining the maximum capacity and further modeling of the kinetics of the system. Langmuir isotherm is shown in Equation 9.3:

$$q_e = \frac{q_m \cdot K_L \cdot C_e}{1 + K_L \cdot C_e} \tag{9.3}$$

q_e [mg/g] is equilibrium adsorbed amount of metal ions, γ_e [mg/l] is equilibrium concentration of metal ions in solution, q_m [mg/g] is maximum adsorption capacity, and K_L [l/mg] is parameter in Langmuir isotherm.

Freundlich isotherm is shown in Equation 9.4:

$$q_e = K_F \cdot C_e^{1/n} \tag{9.4}$$

K_F [l/g] is parameter in Freundlich isotherm and n is parameter in Freundlich isotherm.

Langmuir-Freundlich isotherm is shown in Equation 9.5:

$$q_e = \frac{q_m \cdot K_C \cdot C_e^{1/n}}{1 + K_C \cdot C_e^{1/n}} \tag{9.5}$$

K_C [l/mg] is a parameter in the Langmuir-Freundlich isotherm.

Redlich-Peterson isotherm is shown in Equation 9.6:

$$q_e = \frac{K_{RP} \cdot C_e}{1 + A \cdot C_e^{\beta}} \tag{9.6}$$

K_{RP} [l/g] is parameter in Redlich-Peterson isotherm, and A [l/mg] and β are parameters in Redlich-Peterson isotherm.

The results of the tested system Cr(VI) ions-clayey diatomite using these four models are shown in Fig. 9.11.

The parameters and the coefficients of correlation of the used isotherms, determined by MATLAB/Curve Fitting Toolbox, are listed in Table 9.3.

High values of the correlation coefficients ($R^2 > 0.99$) for Langmuir, Langmuir-Freundlich, and Redlich-Peterson isotherms show good correspondence to the experimental data. Better results for describing the equilibrium of the process for the system Cr(VI) ions-clayey diatomite are given by the Redlich-Peterson isotherm model.

9.3.9 Kinetic of the System Cr(VI) Ions-Clayey Diatomite

To design the adsorption process, besides adsorption capacity or equilibrium tests, it is necessary to examine the kinetics of adsorption process. Research in the field of kinetics move toward finding the most appropriate mathematical model to describe the experimentally obtained kinetic curves of the analyzed system. The kinetics of

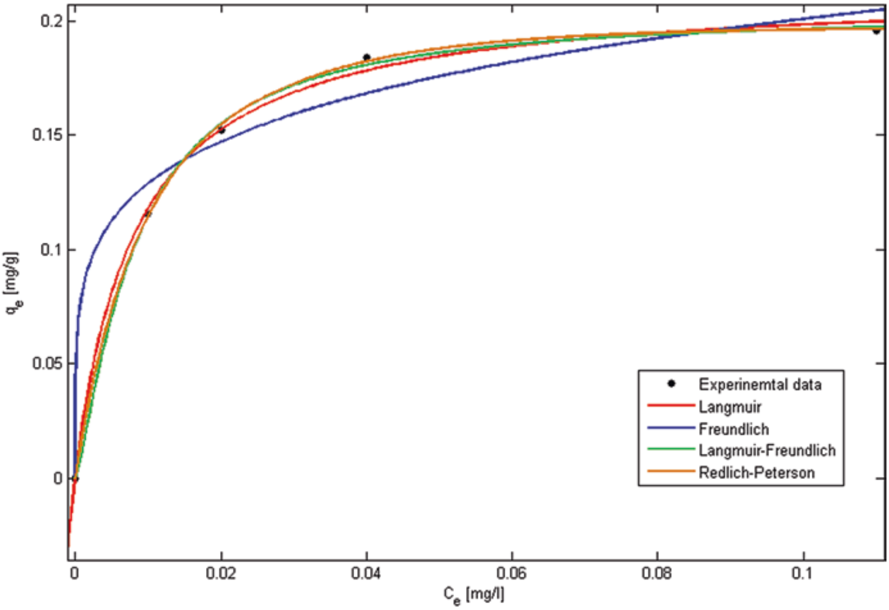

Fig. 9.11 Modeling of the experimental data for the system Cr(VI) ions-clayey diatomite

Table 9.3 Parameters and coefficients of correlation of equilibrium isotherm models

Isotherm	Parameters and coefficients of correlation	Clayey diatomite
Langmuir	q_m [mg/g]	0.2146
	K_L [l/mg]	122.4
	R^2	0.9979
Freundlich	K_F [l/g]	0.3133
	n	5.174
	R^2	0.9796
Langmuir-Freundlich	q_m [mg/g]	0.2044
	K_C [l/mg]	469.5
	n	0.7804
	R^2	0.9991
Redlich-Peterson	K_{RP} [l/g]	20.25
	A [l/mg]	113.8
	β	1.086
	R^2	0.9996

the process of adsorption depends on the physical properties of adsorbent, which in turn have a major impact on the mechanism of adsorption, chemical properties of the system adsorbate-adsorbent, equilibrium behavior, and working conditions in the investigated system.

9.3.9.1 Kinetic Models

Experimental data are analyzed using several kinetic models to determine which model equation describes the kinetic process: pseudo-first- and pseudo-second-order models and model of Elovic.

Model for reaction of pseudo-first order:

$$\ln\left(q_\mathrm{e} - q_\mathrm{t}\right) = \ln q_\mathrm{e} - k_1 t \tag{9.7}$$

k_1 is constant speed of reaction of pseudo-first order [min^{-1}].

- Model reaction of pseudo-second order of reaction:

$$\frac{t}{q_\mathrm{t}} = \frac{t}{q_\mathrm{e}} + \frac{1}{k_2 q_\mathrm{e}^2} \tag{9.8}$$

k_2 is equilibrium constant of the reaction speed of the pseudo-second order [g/mg•min]; $k_2 q_e^2$ or h [mg/g•min] is the initial speed of adsorption.

- Model of Elovic:

$$q_\mathrm{t} = \frac{1}{\beta}\ln\left(\alpha\beta\right) + \frac{1}{\beta}\ln t \tag{9.9}$$

α constant (mg/g•min) is designated by q_t graph regarding lnt, and constant β (g/mg) of the graph is relative to q_t, lnt.

9.3.9.2 Determination of the Adsorption Kinetic Using Pseudo-First-Order Kinetic Model

Obtained experimental data for system Cr(VI)-clayey diatomite analyzed by pseudo-first-order reaction are shown in Fig. 9.12.

9.3.9.3 Determination of the Adsorption Kinetic Using Pseudo-Second-Order Kinetic Model

The obtained experimental data for the system Cr(VI) ions-diatomite analyzed using the pseudo-first-order reaction are shown in Fig. 9.13. Pseudo-first- and pseudo-second-order adsorption parameters are shown in Table 9.4.

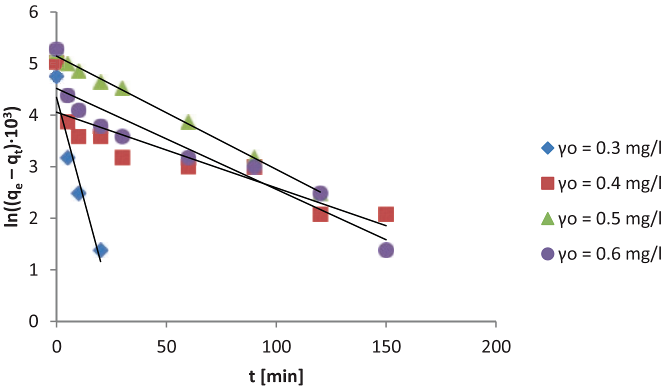

Fig. 9.12 Kinetic adsorption of pseudo-first-order model for the system Cr(VI) ions-clayey diatomite

Fig. 9.13 Kinetic adsorption of pseudo-second-order model for the system Cr(VI) ions-diatomite

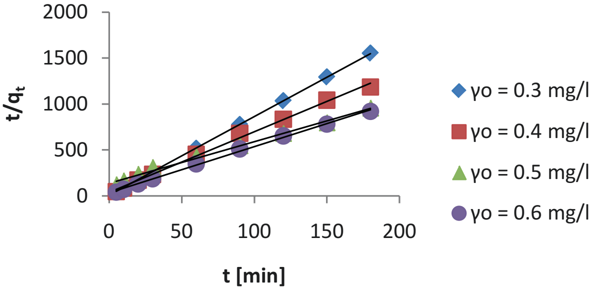

Table 9.4 Parameters derived from pseudo-first- and pseudo-second-order kinetic models

Cr(VI)		Pseudo-first order			Pseudo-second order			
γ_0 [mg/l]	$q_{e,exp}$ [mg/g]	$q_{e,cal.}$ [mg/g]	k_1 [min^{-1}]	R^2	$q_{e,cal.}$ [mg/g]	k_2 [g/mg•min]	h [mg/g•min]	R^2
0.3	0.116	0.077	0.159	0.928	0.116	10.6339	0.145	1.000
0.4	0.152	0.058	0.014	0.777	0.151	1.1223	0.026	0.996
0.5	0.188	0.171	0.021	0.996	0.221	0.1487	0.007	0.994
0.6	0.196	0.092	0.019	0.893	0.199	0.490	0.030	0.997

The values for the coefficients of correlation for pseudo-first-order model are satisfactory but do not necessarily mean that the kinetic reaction takes place by this mechanism. But for the reaction of pseudo-second order, excellent results for the correlation coefficients are obtained. All coefficients have a value above 0.99, indicating that Cr(VI) ion adsorption on clayey diatomite followed pseudo-second-order kinetic model.

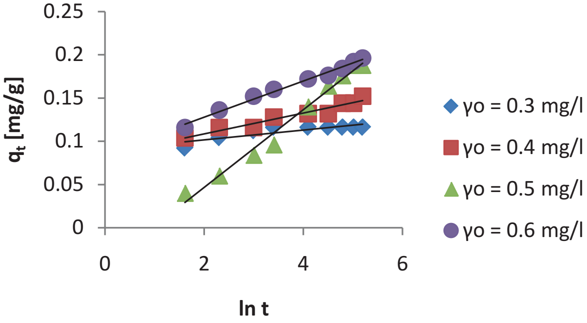

Fig. 9.14 Kinetic model of Elovic for the system Cr(VI) ions-diatomite

Table 9.5 Parameters derived from kinetic model of Elovic

Cr(VI)	Model of Elovic		
γ_0 [mg/l]	α [mg/g•min]	β [mg/g]	R^2
0.3	$32.8 \bullet 10^4$	200.00	0.734
0.4	13.16	83.33	0.939
0.5	0.1143	22.73	0.983
0.6	1.4740	50.00	0.989

9.3.9.4 Determination of the Adsorption Kinetic Using Kinetic Model of Elovic

Obtained experimental data for the system Cr(VI) ions-diatomite analyzed using the kinetic model of Elovic are shown in Fig. 9.14. Kinetic parameters for the model of Elovic are shown in Table 9.5.

The high values of the coefficients of correlation of the Elovic model point out that the adsorption of Cr(VI) ions using diatomite as adsorbent is a reversible process.

9.4 Conclusions

The adsorption of Cr(VI) ions onto a clayey diatomite was shown to occur very efficiently with 96.7%. During the experiments the effect of operating parameters, such as amount of adsorbent (0.5–5.5 g/l), initial adsorbate concentration (0.3, 0.4, 0.5, and 0.6 mg/l), and time of adsorption (5–180 min) at pH of the solution 3, was examined. It was found that 2.5 g/l is optimal adsorbent dosage for maximal removal of Cr(VI) ions. The percentage of removal decreases from 96.7% to 81.7% as initial metal ion concentration increases from 0.3 to 0.6 mg/l, respectively. 45–60 min of adsorption was sufficient time to achieve equilibrium for initial concentrations of

Cr(VI) ions of 0.3 and 0.4 mg/l, while it took 120–150 min to reach the steady state for the concentrations of 0.5 and 0.6 mg/l. The equilibrium of adsorption for the system Cr(VI)-diatomite was analyzed using the following adsorption isotherms: Langmuir, Freundlich, Langmuir-Freundlich, and Redlich-Peterson. Best results to describe the equilibrium of the investigated system are obtained by simulation in MATLAB/Curve Fitting Toolbox using Redlich-Peterson isotherm. To define the kinetics of the studied system, the following models are applied for studying the kinetics of adsorption system Cr(VI) ions-diatomite: model of reaction of pseudo-first and pseudo-second order and model of Elovic. According to the detailed analysis of the applied kinetic models, the best results in terms of describing the kinetics of adsorption process are provided by models of pseudo-second order and Elovic that indicate reversible reaction.

The used natural raw material clayey diatomite from North Macedonia represents a very efficient adsorbent for removal of Cr(VI) ions from aqueous solutions, and, so far, it has not been used in the literature for this purpose which gives to this work originality in scientific and applicative aspect.

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Chapter 10
Chemometric Determination of Macro- and Microelements in Barley Genotypes with Different Origin Grown in the Republic of North Macedonia

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Abstract The main aim of this research was to determine the content of macro- and microelements in barley genotypes, with different origin and spike morphology grown under agroecological conditions in the Republic of North Macedonia. The field experiments were conducted during 2012/2013 and 2013/2014 on the research fields of the Faculty of Agriculture, "Goce Delchev" University, in Ovče Pole. The trials were arranged as randomized complete block design with three replications for each genotype. 30 winter barley genotypes with different origin were used as an experimental material. Five of them are domestic (Hit, Izvor, Egej, Line 1, and Line 2), two varieties are from Croatia (Zlatko and Rex), five genotypes have Serbian origin (NS 525, NS 565, NS 589, Somborac, and Javor), four genotypes are from Hungary (Kompolti korai, Petra, GK Stramm, and GK Judy), two genotypes have Romanian origin (Liliana and Univers), and the remaining are from Bulgaria (Obzor, Perun, Emon, Lardeya, Orfej, Imeon, Zagorec, Asparuh, Kuber, Sajra, Devinija and Odisej). From all tested barley varieties, six cultivars have six-row spike morphol-

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ogy. Microwave digestion method was used to destroy the organic matrix to determine the content of macro- and microelements. The content of all elements was carried out by mass spectrometry with inductively coupled plasma (ICP-MS). The data demonstrated considerable variations in macro- and microelements in barley genotypes. Two-row barley genotypes contained significantly larger levels of tested elements compared to varieties which have six-row spike morphology, except for calcium. From the other side, Bulgarian varieties had lower calcium content compared to other two-row varieties. NS 525 variety showed the highest levels of sodium, cooper, and nickel, while the highest content of phosphorus and zinc was obtained in NS 589. Macedonian genotype Line 2 had the highest concentration of manganese and in Hit variety was established the highest level of aluminum and iron. The differences in grain chemical content may be utilized in barley breeding programs particularly to produce varieties for specific purpose and feed but also to improve chemical composition and grain quality.

Keywords Barley · Variety · Macro- and microelements · Mass spectrometry

10.1 Introduction

Cereals are the most important food source for human consumption. In the last years, cereals have been recognized as functional food because of their mineral composition, antioxidant properties, and fiber content which can provide beneficial effect on consumers and health. Many epidemiological researches have shown that diets rich in whole grain are associated with a decreased risk of a number of chronic diseases such as diabetes II, heart diseases, and certain types of cancers (Susan et al. 2013). Food rich in dietary fiber tend to be a rich source of many other health components such as minerals and antioxidants (Hashemi 2015). The human food needs to be rich not only in macronutrients like protein and carbohydrates but also in micronutrients. According to Anonymous (2013), from approximately 2.3 billion tons of cereals produced, roughly 1 billion tons are used as a source of human food.

10.1.1 Barley: Taxonomy, Origin, and Production

Barley (*Hordeum vulgare* L.) belongs to the genus *Hordeum* in the grass family Poaceae (also known as Graminaceae). There are 32 species within the *Hordeum* genus. Barley is a self-pollinating diploid with $2n = 2x = 14$ chromosomes. *Hordeum vulgare* L. has centers of diversity in central and southwestern Asia, western North America, southern South America, and in the Mediterranean (Von Bothmer 1992). *Fertile Crescent of the Middle East consisting of Turkey, Iran, Iraq, and Lebanon has been reported as the original area of cultivation and most likely origin of barley, the most ancient crop of cereals* (Harlan 1920). *According to the excavations, barley was* *domesticated in the Nile River Valley of Egypt at least 17,000 year ago* (Wendorf et al. 1979). *Barley has an extremely wide geographic range, wider than almost every other crop species. It is cultivated at the highest arable mountaintops down to the seacoast and from the highest north/south latitudes to the tropics (*Paulitz and Steffenson 2011*). Barley is commonly cultivated for centuries due to its versatility, ability to adapt to unfavorable environmental conditions. Hordeum vulgare L. is* one of the oldest and the most important cereal grown worldwide that contains a lot of nutrients. This crop is still considered one of the four most important cereals in the world after wheat, maize, and rice (Akar et al. 2004; Zaefizadeh et al. 2011; Biel and Jacyno 2013).

World barley production trend from 1961 to 2000 with an average of a 10-year period is summarized in Table 10.1 (Akar et al. 2004). If it is compared in terms of area harvested, production, and yield-level criteria, in spite of the fact that there is some decline in terms of area harvested (18%), both production and yield level have increased by 33% and 61%, respectively.

According to Food and Agriculture Organization (FAO 2010), barley is grown on approximately 56 million hectares in the world (average 2006–2008). The FAO records an average production per year for the same period of 143.4 million tons.

Barley is produced in more than 100 countries (FAO 2016). The largest producer country was the Russian Federation with 17,992,517 tons, followed by Germany with 10,730,500 tons (Table 10.2). The Russian Federation is also the leading country in the world in barley-sown area with 8,133,765 ha. In Table 10.2 are presented the 10 leading countries in barley production in the world (FAO 2016). Seven out of ten leading countries in barley production are in Europe and Eurasia (Russian Federation, Germany, France, Ukraine, Spain, Turkey, and the United Kingdom),

Table 10.1 Barley production trends in the world from 1961 to 2000 (Akar et al. 2004)

Years	Area harvested (ha)	Production % of first period	(tons)	% of first period	Yield (kg/ha)	% of first period
1961–1965	68,071,000	100	99,716,000	100	1465	160
1978–1980	84,818,000	124	167,627,000	167	1978	135
1998–2000	55,778,000	82	132,393,000	133	2374	161

Table 10.2 The ten leading countries in barley production in the world (FAO 2016)

Country	Sown area (ha)	Total production (t)	Yield (kg/ha)
Russian Federation	8,133,765	17,992,517	2212
Germany	1,605,000	10,730,500	6685
France	1,899,615	10,306,008	5425
Ukraine	2,859,200	9,435,710	3300
Australia	4,107,648	8,992,274	2189
Canada	2,336,800	8,704,300	3724
Spain	2,800,628	7,979,590	2849
Turkey	2,700,023	6,700,000	2481
United Kingdom	1,122,000	6,655,000	5931
United States of America	1,035,200	4,338,850	4191

Table 10.3 Balkan countries in barley production (FAO 2016)

Country	Sown area (ha)	Total production (t)	Yield (kg/ha)
Bulgaria	159,830	689,850	4316
Greece	151,953	405,696	2637
Serbia	91,530	395,501	4321
North Macedonia	41,297	144,832	3507
Slovenia	19,184	91,653	4777
Bosnia and Herzegovina	25,631	77,294	3015

Table 10.4 Barley sown area, total production, and yield in the Republic of North Macedonia (FAO 2019)

Year	Sown area (ha)	Total production (t)	Yield (kg/ha)
2019	44,098	138,453	3151
2018	44,772	130,028	3072
2017	43,973	93,666	2138
2016	41,297	144,832	3507
2015	41,763	101,677	2470
2014	41,202	153,055	3719

two of them (the United States and Canada) are in North America, and the last one is in Oceania (Australia). In 2016, the highest barley grain yield was observed by Germany (6685 kg/ha), followed by the United Kingdom (5931 kg/ha).

In 2019, also the Russian Federation is the top country in barley production with 19,939,000 tons which accounts for 21.42% of the world's barley production. The Russian Federation was followed by Canada (10,383,000 tons), Ukraine (9,528,000 tons), Australia (8,800,000 tons), and Turkey (7,900,000 tons). Those five countries account for 59.98% of it.

In Table 10.3 are represented the countries with the largest barley production in the Balkan Peninsula. According to the FAO (2016), the first place belongs to Bulgaria with 689,850 t barley production, followed by Greece (405,696 t) and Serbia (395,501 t). The average grain yield in the Republic of North Macedonia was 3507 kg/ha, with 41,297 ha sown area and 144,832 t total barley production (Table 10.3).

In the Republic of North Macedonia, barley production takes the second place after wheat. In Table 10.4 are given the sown area (ha), total production (t), and barley grain yield (kg/ha) in the Republic of North Macedonia in the last 6 years (FAO 2019). In the last 3 years, there is a slight trend of increasing barley production and grain yield.

10.1.2 Barley: Morphology, Vegetative Growth, and Uses

Barley is an annual grass that stands usually from 60 cm to 120 cm tall. Barley has two types of root systems, seminal and adventurous. The stem is erect and made up of hollow, cylindrical internodes, separated by the nodes which bear the leaves (Gomez-Macpherson 2001). A mature barley plant consists of a central stem and 2–5 branch stems, called tillers. The apex of the main stem and each fertile tiller carries a spike. Barley leaves are linear and 5–15 mm wide and are produced on the alternate side of the stem. The leaf structure consists of the sheath, blade, auricles, and ligule. The sheath surrounds the stem completely. The ligule and auricles distinguish barley from other cereals as they are smooth, envelope the stem, and can be pigmented with anthocyanins (Gomez-Macpherson 2001).

Barley is one of the most genetically diverse cereal which is categorized as spring or winter types, hulled or hulless by the presence or absence of hull tightly adhering to the grain (Anderson et al. 2012). Barley comes in two varieties, distinguished by the number of rows of flowers on its flower spike. Six-row barley has its spike notched on opposite sides, with three spikelets at each notch, each containing a small individual flower, or floret, that develops a kernel. Two-row barley has central florets that produce kernels and lateral florets that are normally sterile. In some studies, there were no obtained significant differences between two-row and six-row varieties if grain chemical composition was compared (Aman et al. 1985; Aman and Newman 1986). Two-row varieties are commonly used for malting production because they contain more protein, starch, and crude fat and less crude fiber than six-row type of varieties, which is more suited for animal feed (Day and Dickson 1957; Kong et al. 1995; Marquez-Cedillo et al. 2000). Cultivated barley is a grass that may be either a winter or spring annual. In the Republic of North Macedonia, barley is mainly grown as winter two-row cultivar. On the national variety list, besides winter forms of barley, only one variety (Makedo) is registered as a spring domestic variety (MAFWE 2008).

Barley vegetative growth can be divided into a number of stages: germination, seedling development, tillering, stem elongation, heading, flowering, and ripening. The duration of different developmental stages varies widely. Growth rate depends on the weather, water supply, soil fertility, the degree of competition with other plants, the presence of pests and diseases, and the time of planting.

Barley production is multistage, because it's used for animal feed, producing malt, seed, human food, and as a cover crop to improve soil quality (Gallegos-Infante et al. 2010; Babaeian et al. 2012; Shar et al. 2013). The increased interest in barley as a human food ingredient results from studies that have shown barley to be an excellent source of dietary fiber especially β-glucan. About 2% of the global barley production is used as food (Gupta et al. 2010).

10.1.3 Barley: Grain Composition

Starch is the most abundant component of the barley endosperm, comprising around 60% of total grain weight. Proteins account from 8% to 13% (dry basis) of the total composition of malt barley, and the level of lipids is approximately 2–4% of total grain weight (Welch 1978). Barley grain contains the highest amount of fat-soluble vitamin E, comparing with major cereals (Kerckhoffs et al. 2002).

One of the main goals in the selection of cereal grain is determination of mineral composition, especially phosphorus, potassium, magnesium, calcium, zinc, and essential amino acids for production of functional food (Nardi et al. 2003; Sidhu et al. 2007). The content of macro- and microelements in cereals depends by many factors such as chosen variety, soil and weather conditions during the growing period, and use of fertilizers (Pietola and Salo 2000; Bálint et al. 2001; Hattori and Chino 2001). According to Ficco et al. (2009), the major source for variation in micronutrients in wheat seed is the genotype and environmental interactions.

The previous studies for determination of macro- and microelements have been based on soil, water quality, and air pollution (Klavins and Vircavs 2001; Nikodemus et al. 2004; Tabors et al. 2004; Gilucis 2007; Cekstere and Osvalde 2013). Soil is the main source of trace elements for plaints, both of micronutrients and pollutants (Kabata-Pendias 2011). Most of the researches addressing the issue of mineral content in organic grain have focused on wheat (Zörb et al. 2009; Hussain et al. 2010). But in the last years, many papers are dedicated to macro- and microcontent in barley varieties (Šterna et al. 2015; Markova Ruzdik et al. 2016; Jâkobsone et al. 2018).

Minerals which affect the nutritive value of barley seed are divided into macro- and microelements based on concentration in food. The macroelements include calcium, phosphorus, potassium, magnesium, and sodium. The rest are iron, manganese, zinc, selenium, and cobalt which are the nutritionally microelements in barley kernel (Marconi et al. 2000).

From the nutritional point of view, attention is mainly focused on essential macroelements (K, Ca, and Mg) and trace elements (Fe, Cu, Mn, and Zn) in grains. The products of cereals provide significant amounts of the most nutrients including relevant quantities of minerals. Minerals in cereal grain are mostly found in the aleurone layer (Lui et al. 2007; Poutanen 2012). Even though micronutrients are needed in minute quantities, they have major impact on normal development. The amount of essential heavy metals such as Ni, Cu, and Zn in agricultural products has been reported previously (Pirsaheb et al. 2015).

On the other side, mineral nutrition contributed significantly to increase crop yields. Borlaug and Dowswell (1994) presented that around 50% of the increase in crop yields worldwide in the last period was due to application of chemical fertilizers. Crops need micronutrient elements in small quantities, for normal growth and production. Deficiencies of essential elements cause disturbance in the physiological and metabolic processes in plants (Bacha et al. 1997). Plant growth can be limited because of low content of the following elements: nitrogen, phosphorus, potassium, calcium, magnesium, iron, and molybdenum (Rao et al. 1993; Samac and Tesfaye 2003). The content of calcium is low in maize and barley, but barley is the first crop according to content of phosphorus, compared to other cereals (Shar et al. 2013). According to Welch et al. (1991), the elements manganese, iron, copper, zinc, boron, and molybdenum are essential for higher plants, while Parker et al. (1992) reported that zinc is of the eight essential elements, necessary for normal growth and plant development.

A lot of barley breeding programs have as main objective to develop new genotypes that have some improvement over existing variety traits like yield potential, resistance to biotic and abiotic stress factors, richer content of macro- and micronutrients, as well as proper end-use quality. Also, one of the main barley breeding objectives is creation of barley varieties suitable for malt and beer production (Matthies et al. 2014) because the key to success in the seed and seed market for malt and beer industry depends on quality parameters of barley variety (Križanova et al. 2010).

The aim of this study was to determine the content of some macro- and microelements in barley genotypes, with different origin, grown under environmental condition in the Republic of North Macedonia and to evaluate the differences in mineral composition in two- and six-row varieties if they exist.

10.2 Materials and Methods

10.2.1 Geographic Characterization of the Investigated Area

The investigated trial was carried on a research area in Ovče Pole valley, near Sveti Nikole municipality, in the east-central part of the Republic of North Macedonia (Fig. 10.1). Ovče Pole is a plain situated around the flow of Sveti Nikole's river, which is a tributary to the Bregalnica River, within the following geographic coordinates N: 41°49′21.9″ and E: 21°59′03.9″. The average elevation of the plain is

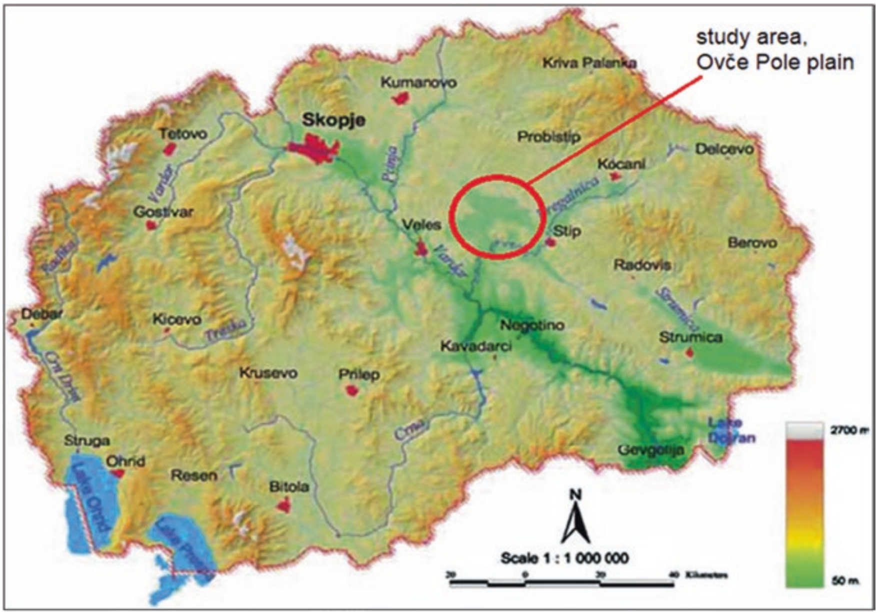

Fig. 10.1 The investigated area on the territory of the Republic of North Macedonia

200–400 m and the highest place is Gjurište, with an elevation of 856 m above sea level. It's a stone's throw away from the big conglomerates of Skopje, Veles, Štip, and Kumanovo.

The climate in Ovče Pole valley is characterized by hot and dry summers and temperately cold winters, with occasional sharp lows. The highest registered temperature in the plain was 44.0 °C and the lowest registered temperature was −23.0 °C. According to Filipovski et al. (1996), the Republic of North Macedonia is divided on eight climatic vegetation areas. Based on this classification, the experimental area belongs to the continental sub-Mediterranean area. Strong winds from the northwest, north, and southeast are specific for this region and are present for most of the year. That's why this area is called "the windiest place on the Balkans." The Ovče Pole valley is one of the driest areas in Europe and is plagued by frequent drought periods. The yearly average of rainfall ranges from 400 to 500 mm/m^2.

10.2.2 Field Experimental Design, Soil, Climate, and Agronomic Practice

The investigated area on which the research was conducted is owned by the Faculty of Agriculture, "Goce Delchev" University of Štip, the Republic of North Macedonia. The field experiments were set up during two consecutive growing seasons (2012–2013 and 2013–2014). Randomized block system, with three replications for each genotype, was used for field research. Each replication plot was 1 m^2, consisted of 10 rows. The seedling density was calculated on the basis of 500 germinated seeds per m^2. Standard agronomic practices were applied during the vegetation period.

Before sowing, agrochemical soil analysis was performed. From the experimental plot were taken samples for soil analysis. The results from analysis showed that the soil was good supply with readily available phosphorus and total nitrogen, rich in easily available potassium, low humus content, and low salinity (Table 10.5).

The average air temperature and precipitations differ during the period of cultivation in both experimental years (Table 10.6). The most significant differences in temperatures were noticed in February: 15.3 °C in the first experimental year and 7.0 °C in the second testing year. Although the amount of precipitation during both years didn't differ much, there were more significant differences in the distribution of precipitation by months.

Table 10.5 Agrochemical analyses of soil samples

Available P_2O_5 (mg/100 g soil)	Available K_2O (mg/100 g soil)	Humus (%)	Total N (mg/g)	pH	K (mg/kg)	P (mg/kg)	Fe (mg/kg)	Na (mg/kg)
24.19	74.1	1.94	0.98	7.65	2.18	827	4.86	1.19

Table 10.6 Average air temperature and precipitation sum during the experimental period

	Average temperature (°C)		Precipitation sum (mm)	
Month	2012–2013	2013–2014	2012–2013	2013–2014
October	16.6	13.0	40.6	11.8
November	10.5	9.1	37.4	65.8
December	1.5	0.3	33.7	20.3
January	2.4	3.9	33.6	42.1
February	15.3	7.0	119.3	12.8
March	9.1	9.6	35.0	25.0
April	13.2	12.2	63.2	148.2
May	18.6	16.6	44.8	59.3
June	21.7	20.3	23.8	69.3
Average	12.1	10.2	431.4	454.6

Barley genotypes were sown by hand, 3–5 cm deep, on 18 October in the first experimental year. In the second testing year, the sowing was done on 14 October. Pre-sowing soil preparation was conducted in suitable timing, in both years and in accordance with weather conditions. The barley genotypes were harvested manually in June in both years.

10.2.3 Plant Material

Thirty winter barley genotypes were used as experimental material with different origin. Six from them are six-row, and the other barley varieties have two-row spike morphology (Table 10.7). In Table 10.7 are presented the used barley genotypes with their origin and spike morphology. Five of them are from the Republic of North Macedonian, five genotypes have Serbian origin, two genotypes are from the Republic of Croatia, four genotypes are from the Republic of Hungary, two genotypes have Romanian origin, and the remaining ten genotypes have Bulgarian origin (Table 10.7).

10.2.4 Sample Preparation and Applied Methods

Determination of macro- and microelements was performed in laboratory "UNILAB," Faculty of Agriculture, "Goce Delchev" University of Štip, Republic of North Macedonia. For digestion of barley grain samples, the microwave digestion system (model Mars, CEM) was applied (Fig. 10.2). Precisely 0.5 g of grind barley grain was measured and placed in Teflon digestion vessels from each genotype. 5 ml concentrated nitric acid, HNO (69%, 108 m/V), and 2 ml hydrogen peroxide,

Table 10.7 Origin and spike morphology of winter barley genotypes used in the research

Genotype	Origin	Spike morphology
Hit	North Macedonia	Two-row
Izvor	North Macedonia	Two-row
Egej	North Macedonia	Two-row
Line 1	North Macedonia	Two-row
Line 2	North Macedonia	Two-row
Zlatko	Croatia	Two-row
Rex	Croatia	Two-row
NS 525	Serbia	Two-row
NS 565	Serbia	Two-row
Obzor	Bulgaria	Two-row
Perun	Bulgaria	Two-row
Emon	Bulgaria	Two-row
Lardeya	Bulgaria	Two-row
Orfej	Bulgaria	Two-row
Imeon	Bulgaria	Two-row
Zagorec	Bulgaria	Two-row
Asparuh	Bulgaria	Two-row
Kuber	Bulgaria	Two-row
Sajra	Bulgaria	Two-row
Devinija	Bulgaria	Two-row
Odisej	Bulgaria	Two-row
NS 589	Serbia	Two-row
Somborac	Serbia	Six-row
Javor	Serbia	Six-row
Kompolti korai	Hungary	Two-row
Petra	Hungary	Six-row
GK Stramm	Hungary	Six-row
GK Judy	Hungary	Two-row
Liliana	Romania	Six-row
Univers	Romania	Six-row

Fig. 10.2 Sample preparation for decomposition in a microwave oven

Table 10.8 Work program for microwave digestion of barley samples (model Mars, EMS)

Step	Temperature (°C)	Time (min)	Power (W)	Pressure (bar)
1	180	5	500	20
2	180	10	500	20

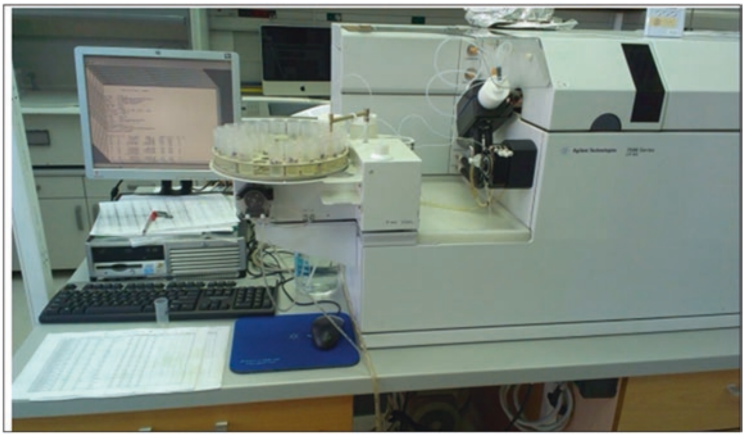

Fig. 10.3 Mass spectrometry with inductively coupled plasma (ICP-MS)

H_2O_2 (30%, m/V), were added. The Teflon vessels were carefully closed and placed in microwave for digestion.

Barley samples were digested in two steps for total dissolving at 180 °C (Table 10.8). After the digestion method was finished, digested samples were quantitatively transferred into 25 ml volumetric flaks.

The obtained solutions were used for chemical analysis. Concentrations of ten macro- and microelements (Na, Mg, Al, P, Ca, Mn, Fe, Cu, Zn, and Ni) were determined by mass spectrometry with inductively coupled plasma (ICP-MS) (Fig. 10.3).

10.2.5 Statistical Data Processing

Data collected from 2 years of experiment was subjected to variance analysis using JMP (2002) statistical software. Fit analysis was performed to obtain the least significant differences (LSD values) for tested genotypes by different elements. Based on the LSD data, the genotypes were statistically processed, grouped, and marked with alpha/beta characters, after the value for each element content. Genotypes with the highest values of the studied element belong in group a. All genotypes which statistically differ between each tested element are marked by different alpha/beta character. LSD test allows to test different cultivars for different elements (traits) and show the statistical differences between tested cultivars if they exist. This test is often used in agricultural practice, especially in breeding programs, and helps breeders to choose the varieties with different mineral content and use them as a parent in order to create the variety with desired traits. The values for mineral content were statistically processed using basic descriptive statistics (SPSS 2010). To determine the variability of analyzed elements and understand the connection between metal contents, principal component analysis (PCA) and cluster analyses (CA) were applied (Mohammadi and Prasanna 2003).

10.3 Results and Discussion

Minerals are inorganic elements found in small or insignificant amounts in various dietary constituents. Based on the amount present in the cell, minerals are classified as major or macro and trace minerals. The major elements are those that are present in the cell in amounts greater than 5 g. Trace minerals are those present in amounts less than 5 g (Kulp and Ponte 2000). Main and trace elements have many functions: as electrolytes, as enzyme constituents, and as biding materials in bones and teeth.

The barley grain is a source of minerals for human and livestock nutrition. According to Shwry and Ullrich (2014), typical components of nutritionally important minerals (including iron and zinc, which are deficient in many human diet) in whole grain and barley samples are phosphorus (3560μg/g), potassium (5340μg/g), magnesium (1450μg/g), calcium (520μg/g), iron (46μg/g), zinc (31μg/g), manganese (20μg/g), and copper (7μg/g).

The average mineral content in different barley varieties varies significantly from one part of the world to another. Mineral content in barley grain is a function of factors such as type of grain, the variety agricultural procedures, growing conditions, composition of soil, and harvest time (Potter and Hotchkiss 1995; Fennema 1996; Kulp and Ponte 2000).

The results obtained for macro- and microelements in tested barley genotypes are summarized in Tables 10.9 and 10.10. Using LSD test, significant differences were noticed between analyzed varieties among different elements. The differences in chemical composition in barley cultivars can be explained by genetic makeup since all genotypes were grown under the same environmental conditions. Kabata-Pendias (2011) have been reported that the macro- and trace element contents of plants are affected by the cultivar, soil conditions, weather conditions during the period of growth, and use of fertilizers.

Sodium (Na) is an important mineral and is present mostly as an extracellular constituent. The function of this mineral is mainly to maintain the osmotic pressure of the extracellular fluid. From our study, sodium concentration ranges from 21.57 mg/kg in Emon variety to 90.50 mg/kg in NS 525 cultivar. Significant differences were noticed between tested barley genotypes. The average value for sodium for all analyzed varieties was 33.00 mg/kg with small coefficient of variation (0.96%). No significant differences were obtained between the following

Table 10.9 Average values of Na, Mg, Al, P, and Ca content in analyzed barley genotypes

Genotype	Na (mg/kg)	Mg (mg/kg)	Al (mg/kg)	P (mg/kg)	Ca (mg/kg)
Hit	31.59 k	427.20 e	9.01 a	851.0 e	117.35 i
Izvor	48.27 b	394.20 l	7.86 b	787.5 o	115.25 j
Egej	32.68 j	387.25 p	3.63 mn	834.5 i	96.05 s
Line 1	31.85 k	411.40 j	3.34 pq	768.5 q	104.10 n
Line 2	24.36 op	437.25 a	4.32 h	912.5 b	103.55 n
Zlatko	35.44 h	384.85 q	3.90 jkl	872.0 c	121.15 g
Rex	34.42 i	378.30 s	4.05 ij	734.0 u	115.15 j
NS 525	90.50 a	420.95 f	3.82 l	836.5 h	120.90 g
NS 565	23.35 r	395.80 k	3.59 no	756.0 s	99.10 q
Obzor	35.61 gh	389.15 o	5.20 d	778.5 p	111.00 l
Perun	27.43 n	375.05 v	3.19 q	761.0 r	120.60 g
Emon	21.57 s	417.00 h	3.85 kl	840.0 g	113.80 k
Lardeya	24.70 o	434.65 c	2.98 r	744.0 t	94.70 t
Orfej	32.98 j	394.10 m	4.00 ijk	801.5 m	117.90 i
Imeon	41.03 d	389.55 n	5.00 e	792.5 n	97.65 r
Zagorec	39.76 e	377.20 t	4.08 i	825.5 k	101.50 o
Asparuh	21.75 s	357.45 x	4.63 f	693.0 w	68.50 w
Kuber	41.76 c	417.80 g	3.49 nop	829.5 j	101.40 o
Sajra	31.07 l	412.05 i	4.83 e	844.0 f	85.55 u
Devinija	23.70 qr	342.90 z	3.77 lm	641.5 α	84.65 v
Odisej	30.67 l	430.05 d	4.65 f	862.0 d	111.45 l
NS 589	23.78 qr	435.85 b	4.44 gh	979.0 a	161.65 b
Somborac	23.28 r	372.20 w	4.56 fg	682.0 x	129.05 f
Javor	35.96 g	378.65 r	3.94 ijkl	652.0 z	133.15 e
Kompolti korai	30.15 m	319.95 β	3.46 op	666.5 y	100.40 p
Petra	36.62 f	355.85 y	7.61 c	820.0 l	181.65 a
GK Stramm	21.72 s	323.75 α	2.94 r	707.5 v	136.95 d
GK Judy	36.00 g	375.25 u	2.83 r	849.5 e	106.40 m
Liliana	34.91 i	311.25 γ	2.48 s	547.0 β	119.00 h
Univers	24.15 pq	270.60 δ	3.55 no	514.5 γ	139.00 c
Average	33.00	383.92	4.30	772.8	113.58
LSD$_{0.05}$	0.517	0.087	0.171	1.847	0.647
CV (%)	0.96	0.01	2.43	0.15	0.33

Levels not connected by the same alpha/beta characters, after value for each tested element has significant differences at the $P \leq 0.05$ level

genotypes: Hit and Line 1 which are Macedonian two-row barley genotypes; Egej and Orfej varieties; Rex and Liliana cultivars; NS 565 and Somborac (both varieties have Serbian origin); Emon, Asparuh, and GK Stramm varieties; Devinija and NS 589 cultivars; Sajra and Odisej (both varieties have two-row spike morphology and were introduced from the Republic of Bulgaria); and Javor and GK Judy genotypes.

Magnesium (Mg) is another essential element that has a wide range of roles in many plant functions. This element is a constituent and activator of many enzymes

Table 10.10 Average values of Mn, Fe, Cu, Zn, and Ni content in analyzed barley genotypes

Genotype	Mn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Ni (mg/kg)
Hit	5.59 fg	18.19 a	1.71 efgh	5.12 g	1.570 g
Izvor	6.08 c	16.98 b	1.51 hijklm	4.32 m	0.136 v
Egej	5.27 lm	13.74 jk	1.63 fghi	5.12 g	0.106 w
Line 1	5.62 gh	13.55 k	1.79 bcdef	4.72 j	0.154 t
Line 2	5.56 hi	15.24 f	1.96 abcd	5.77 c	0.702 k
Zlatko	5.95 d	14.68 g	1.68 efgh	3.79 p	0.262 q
Rex	5.74 ef	13.71 jk	1.68 efghi	4.71 j	1.047 h
NS 525	5.81 e	14.68 g	2.06 a	5.57 d	7.743 a
NS 565	5.42 jk	14.11 i	1.67 efghi	4.67j	0.055 z
Obzor	6.19 b	15.86 e	1.70 efgh	5.30 e	6.453 b
Perun	5.31 l	14.05 ij	1.76 defg	5.08 g	0.844 i
Emon	5.99 d	14.12 i	1.81 bcdef	5.02 h	0.709 j
Lardeya	5.50 ij	13.00 l	1.78 bcdef	4.58 k	0.588 l
Orfej	6.11 bc	14.76 g	2.00 abc	5.09 g	1.583 f
Imeon	4.91 o	14.73 g	1.57 fghijk	5.28 e	3.269 e
Zagorec	4.44 rs	16.44 c	1.66 efghi	5.11 g	4.943 c
Asparuh	4.75 p	14.29 hi	1.60 fghijk	4.78 i	0.086 x
Kuber	5.41 k	15.48 f	1.88 abcde	5.56 d	0.323 n
Sajra	5.23 m	16.03 de	1.81 bcdef	5.21 f	0.312 o
Devinija	4.51 r	12.29 mn	1.36 klm	4.09 n	0.143 u
Odisej	6.53 a	16.63 bc	2.02 ab	6.05 b	0.107 w
NS 589	6.15 bc	17.95 a	1.99 abcd	6.38 a	4.133 d
Somborac	4.65 q	12.40 mn	1.53 ghijkl	4.00 o	0.143 u
Javor	4.40 s	12.30 mn	1.66 efghi	4.48 l	0.275 p
Kompolti korai	4.42 s	12.56 m	1.36 jklm	3.71 q	0.0706 y
Petra	5.13 n	16.33 cd	1.60 fghij	5.12 g	0.355 m
GK Stramm	4.40 s	12.18 n	1.27 m	4.69 j	0.231 r
GK Judy	5.52 i	14.52 gh	1.76 cdefg	6.01 b	0.178 s
Liliana	3.77 t	8.72 o	1.44 ijklm	3.60 r	0.143 u
Univers	4.67 q	7.04 p	1.31 lm	3.27 s	0.014 α
Average	5.30	14.22	1.68	4.87	1.223
$LSD_{0.05}$	0.083	0.352	0.242	0.051	0.003
CV (%)	0.10	1.52	8.80	0.65	0.17

Levels not connected by same alpha/beta characters, after value for each tested element has significant differences at the $P \leq 0.05$ level

and stabilizer of plasma membranes, intracellular membranes, and nucleic acids. Magnesium plays a key role in the photosynthesis process, and its deficiency is detrimental to plant growth. In our research the content of magnesium was very different in analyzed barley varieties. Using fit analysis, all tested genotypes were significantly differed (Table 10.9). The concentration of magnesium was the highest in Line 2 (437.25 mg/kg), followed by Serbian variety NS 589 (435.85 mg/kg). The smallest magnesium content was registered in six-row variety Univers (270.60 mg/

kg). Generally, six-row barley genotypes had lower magnesium content compared to two-row barley varieties. Actually, the magnesium content in six-row barley genotypes was lower from the mean data for all tested genotypes. The mean value of magnesium for all tested barley cultivars was 383.92 mg/kg, and the coefficient of variation was very small (0.01).

Aluminum (Al) is an element affecting the growth and grain yield of many crop plants. Yet, some plants are able to accumulate high levels of aluminum. It is considered to be phytotoxic to the majority of plants if the soil pH decreases below 5.5 (Delhaize and Ryan 1995). In this paper, Al concentration ranges from 2.48 mg/kg in Liliana cultivar to 9.01 mg/kg in Macedonian variety Hit. The mean value for aluminum content for all tested varieties was 4.30 mg/kg, and the coefficient of variation was 2.43%. According to LSD value, all genotypes were belonging in different groups, marked with different alpha/beta character, except the following varieties: NS 565 and Univers; Lardeya, GK Stramm, and GK Judy cultivars; Imeon and Sajra (both are Bulgarian varieties with two-row spike); and Asparuh and Odisej cultivars which have two-row spike morphology and Bulgarian origin.

Phosphorus (P) is an essential macroelement for the general health and vigor of all plants. This nutrient is noted especially for its role in capturing and converting the sun's energy into useful plant components. Some specific growth factors that have been associated with phosphorus are stimulated root development, increased stem strength, improved flower formation and seed production, improvements in crop quality, and increased resistance to plant diseases. Phosphorus is also concentrated in the embryo and outer layer of the grain, where it is largely present as phytate. All tested barley varieties significantly differ in phosphorus content except Hit variety and GK Judy, which belong in the same group and didn't differ between them. NS 589 had the highest concentration of phosphorus (979.0 mg/kg), followed by Line 2 (912.5 mg/kg). The average value for phosphorus content in all tested barley varieties was 772.8 mg/kg with small coefficient of variation (0.15%). The lower content of phosphorus showed six-row barley genotypes (Somborac, 682.0 mg/kg; Javor, 652.0 mg/kg; Liliana, 547.0 mg/kg; and Univers, 514.5 mg/kg).

Similar results were presented by Bleidere and Grunte (2008). Those researches examined barley varieties with different origin about chemical composition. The results from their study showed that the content of phosphorus was lower (3.73 g/kg) in six-row barley genotypes compared to two-row (from 3.83 to 5.17 g/kg). From the other side, Bleidere and Grunte (2008) obtained more higher values for phosphorus content compared to results from our research.

Calcium (Ca) is one of the most important macronutrients in plants, with concentration in the shoot ranging from 0.1% to over 5% of dry matter (Marschner 1995). Calcium has dual function, as a structural component of cell walls and membranes and as intracellular second messenger. In our paper, Petra variety has the highest calcium content (181.65 mg/kg), followed by NS 589 (161.65 mg/kg). Generally, Bulgarian varieties had lower calcium content, and the lowest was recorded in Asparuh cultivar (68.50 mg/kg). In this case, six-row barley varieties showed higher values for calcium content, compared to those who had two-row spike. Also, the content of calcium in six-row barley varieties was higher than the mean data for this element for all tested cultivars. No significant differences were noticed between the following barley genotypes: Hit and Orfej; Izvor and Rex; Line 1 and Line 2 (both are two-row Macedonian genotypes); Zlatko, NS 525, and Perun varieties; Obzor and Odisej varieties (Bulgarian cultivars with two-row spike); and Zagorec and Kuber (two-row cultivars with Bulgarian origin). The mean data for all tested barley varieties was 113.58 mg/kg, and the percentage of variation was 0.33% (Table 10.9).

Cioùek et al. (2012) also conducted a study for determination of macro- and microelements in cereal cultivars such as wheat, barley, and oat during 2009. The results from their research showed that barley had 0.393 g/kg calcium and 0.848 g/kg magnesium. Those values were much higher compared to the mean values for same elements in our research.

Even if the concentrations of micronutrients in grains are small, they are also essential for the proper growth and normal plant development. Their accumulation in cereal grain is influenced by multiple factors, including species and soil conditions, organic matter content, pH, and fertilization applied (Wiśniowska-Kielian and Klima 2007).

Duffeus and Roise (1976) also determined the accumulation of minerals within the individual tissues (aleurone, embryo, and pericarp plus testa) during grain development. At 55 days after maturity, about 15–20% of the iron was located in the pericarp, 7–8% in the embryo, and the remainder in the endosperm. The study also showed that a significant increase in the manganese content of the embryo occurred during the later stages of seed development.

Manganese (Mn) is an important metal activator for various enzymes (Belitz et al. 2004). In our research, the differences of manganese concentration between barley genotypes were statistically significant (Table 10.10). Odisej was the variety with the highest content of manganese (6.53 mg/kg), followed by Obzor (6.19 mg/kg). Liliana cultivar had the lowest manganese content (3.77 mg/kg). GK Stramm and Javor also showed low value for manganese content (4.40 mg/kg). No statistically significant differences were obtained between the following varieties: Zlatko and Emon, Orfej and NS 589 cultivars, Somborac and Univers (six-row barley varieties with different origin), and Javor, Kompolti korai, and GK Stramm varieties. The mean data for manganese content for all tested barley varieties was 5.30 mg/kg with small percentage of variation (1.00%). Six-row barely genotypes showed lower values from average data for manganese content but also, in the same time, lower values compared to two-row barley varieties.

According to Cioùek et al. (2012), the mean data for content of manganese in barley varieties was 10.45 mg/kg, and iron and zinc concentrations were 36.08 mg/kg and 32.18 mg/kg, respectively. Those results were much higher compared to the mean data for the same elements obtained in our research.

Iron (Fe) is a very important micronutrient for all living organisms because it plays a critical role in metabolic processes such as DNA synthesis, respiration, and photosynthesis. Further, many metabolic pathways are activated by iron, and it is a prosthetic group constituent of many enzymes. Iron is the third most limiting nutrient for plant growth and metabolism, primarily due to the low solubility of the oxidized ferric form in aerobic environments (Zuo and Zhang 2011; Samaranayake et al. 2012). In plants, iron is involved in chlorophyll synthesis, and it is essential for the maintenance of chloroplast structure and function.

In Table 10.10 are given the iron concentrations in tested barley varieties obtained from the study. No statistical difference was noticed between Hit and NS 589 varieties. Those genotypes had the highest value of iron content (18.19 mg/kg and 17.95 mg/kg, respectively). The lowest iron concentration was recorded in Univers and Liliana cultivars (7.04 mg/kg and 8.72 mg/kg, consequently). Those varieties have the same origin (Romania) and belong to the six-row barley varieties. Generally, six-row cultivars showed lower average value for iron content compared to barley genotypes which have two-row spike morphology. No statistical differences were obtained also between the following barley genotypes: Egej and Rex; Line 2 and Kuber cultivars; Zlatko, NS 525, Orfej, and Imeon varieties; NS 565 and Emon; and Devinija, Somborac, and Javor. The average value for iron content for all analyzed barley varieties was 14.22 mg/kg with 1.52% coefficient of variation.

Ma et al. (2004) determined the iron content of 274 barley cultivars grown on the same site in Japan. The concentration varied widely, from 24.6 to 63.3 mg/g with no relationship to the type of barley (hulled or naked, two-rowed or six-rowed).

Copper (Cu) is a redox-active transition metal essential for plants, as well as for all living organisms. It plays a key role in photosynthetic and respiratory electron transport chains, ethylene sensing, cell wall metabolism, and oxidative stress protection. Plants require Cu for normal growth and development, and when it is not available, plants develop specific deficiency symptoms, most of which affect young leaves and reproductive organs.

From all tested barley varieties, only three genotypes had cooper content higher than 2 mg/kg. NS 525 was the variety with the highest copper concentration (2.06 mg/kg), followed by Odisej (2.02 mg/kg) and Orfej (2.00 mg/kg). The lowest copper content was obtained by GK Stramm (1.27 mg/kg) and Univers (1.31 mg/kg); both genotypes had six-row spike. Using LSD test, significant differences were recorded, except between the following varieties: Hit, Zlatko, and Obzor varieties; Line 1, Emon, Lardeya, and Sajra cultivars; Line 2 and NS 589 genotypes; Rex, NS 565, Zagorec, and Javor varieties; Imeon and Asparuh (Bulgarian varieties with two-row spike); and Devinija and Kompolti korai cultivars. The average copper content for all tested barley genotypes was 1.68 mg/kg with the highest coefficient of variation (8.80%) between tested elements.

Zinc (Zn) is a plant micronutrient which is involved in many physiological functions. Zinc deficiency is the widest spread micronutrient deficiency problem; almost all crops, sandy soils, peat soils, and soils with high phosphorus and silicon are expected to be deficient. Zinc deficiencies can affect plant by stunting its growth, decreasing number of tillers, chlorosis and smaller leaves, increasing crop maturity period, spikelet sterility, and inferior quality of harvested products. Zinc is a component of a number of enzymes. In Table 10.10 are presented the average values for zinc content in all tested barley genotypes. Zinc content ranges from 3.27 mg/kg to 6.38 mg/kg. The average value for all tested barley varieties was 4.87 mg/kg, and the percentage of variance was very small (0.65%). Data showed that genotypes with six-row spike morphology had lower zinc content compared to varieties who had two-row. Significant statistical differences were noticed between tested barley varieties, except between the following genotypes: Hit, Egej, Perun, Orfej, Zagorec, and Petra. On the other hand, the genotypes Line 1, Rex, and GK Stramm belong to the same group which means that no statistical difference was recorded. Also, the genotypes NS 525 and Kuber have similar values for zinc concentration, and they didn't differ between them. The genotypes with high value of zinc, after NS 589, were Odisej and GK Judy (6.05 mg/kg and 6.01 mg/kg, respectively), and they also didn't show difference between them.

According to Newman and Newman (2008), the average values for copper, manganese, iron, and zinc content were 4.98 mg/kg, 19.43 mg/kg, 36.0 mg/kg, and 27.7 mg/kg, respectively, and they were much higher compared to the mean values for the same elements from our research.

The essence of nickel as a micronutrient in plants has been established because it is part of the active site of the enzyme urease, which hydrolyzes urea in plant tissues (Polacco 1977; Eskew et al. 1984). Nickel (Ni) concentration in barley varieties is given in Table 10.10. Nickel content ranges from 0.014 mg/kg in Univers variety to 7.743 mg/kg in NS 525 genotype. Significant differences were obtained between tested barley genotypes, except between Egej and Odisej (both two-row barley varieties). Also, no statistically significant differences were noticed between the following genotypes: Devinija, Somborac, and Liliana. The average value for nickel content in all analyzed barley genotypes was 1.223 mg/kg.

Jâkobsone et al. (2015) investigated the concentration of macro- and trace elements in cereals (rye, barley, wheat, triticale, and oats) cultivated in Latvia. According to this research, the copper and zinc concentrations were very variable in barley (1.71–5.9 mg/kg and 16–53 mg/kg, respectively). The content of nickel (1.088 mg/kg) and sodium concentration (107.4 mg/kg) were the highest in barley cultivars compared to other cereals. Aluminum was 3.242 mg/kg, cooper 3.607 mg/kg, calcium 375.6 mg/kg, iron 37.8 mg/kg, magnesium 1157 mg/kg, and zinc 26.4 mg/kg, while the content of manganese (12.4 mg/kg) was the lowest in barley samples compared to other tested cereals. The data from our research showed that the concentrations of all tested elements were lower compared to results obtained from Jâkobsone et al. (2015) study for all elements except for nickel and aluminum.

In Table 10.11 is given basic descriptive statistics for tested elements in barley genotypes. Magnesium concentration showed the highest range, and the smallest was recorded for cooper element. Sodium concentration ranges from 21.57 mg/kg to 90.50 mg/kg, while Al content varies from 2.48 mg/kg to 9.01 mg/kg. The values of elements P, Ca, Mn, Fe, Zn, and Ni were in the following ranges: 514.50–979.00 mg/kg, 68.50–181.65 mg/kg, 3.77–6.53 mg/kg, 7.04–18.19 mg/kg, 3.27–6.38 mg/kg, and 0.014–7.743 mg/kg, respectively.

Rubene and Kuka (2007) studied eight barley grain samples from different regions of Latvia. According to those authors, the content of phosphorus ranges from 3.30 to 4.10 g/kg, Ca from 0.50 to 0.62 g/kg, Mg from 1.20 to 1.40 g/kg, Na from 43.50 to 49.40 mg/kg, Mn from 14.50 to 19.70 mg/kg, Cu from 4.66 to 6.01 mg/kg, Zn from 18.72 to 30.30 mg/kg, and Fe from 66 to 90.88 mg/kg. If we

Table 10.11 Descriptive statistics for macro- and microelements in barley genotypes

Element	Range	Minimum	Maximum	Mean	Standard mean error	Standard deviation
Na	68.93	21.57	90.50	33.04	2.347	12.86
Mg	166.65	270.60	437.25	383.92	7.358	40.30
Al	6.53	2.48	9.01	4.30	0.270	1.47
P	464.5	514.50	979.00	772.78	18.614	101.95
Ca	113.15	68.50	181.65	113.62	4.081	22.35
Mn	2.76	3.77	6.53	5.30	0.124	0.68
Fe	11.15	7.04	18.19	14.22	0.434	2.38
Cu	0.79	1.27	2.06	1.69	0.039	0.21
Zn	3.11	3.27	6.38	4.87	0.137	0.75
Ni	7.729	0.014	7.743	1.22	0.368	2.02

compare those results with ours, it can be marked that all tested elements in our study had lower values except sodium.

Kan (2015) investigated macroelement (K, P, Ca, Na and Mg) content in cereal cultivars (wheat, barley, ray, triticale, and oat) in Turkey during growing season 2010–2011. Significant differences were reported by tested cereals. Content of P in barley was 3567 ppm, while Mg content 1667 ppm. Fe content was 35 ppm, Mn was 17 ppm, and Cu was 20 ppm. The results from Kan's research were much higher compared with mineral content in samples from our study. Noteworthy, several factors may affect the elemental content: variety, harvesting time, soil type, soil conditions, fertilization, irrigation, weather, etc. (Kan et al. 2005; Ahmet and Bouhadjera 2010).

Eight barley varieties were used as an experimental material in Shar et al.'s (2013) paper. The experiment was conducted in Pakistan during 2010 and analyzed essential trace elements as well as trace and toxic elements such as iron, zinc, manganese, copper, cobalt, chromium, nickel, lead, cadmium, barium, and aluminum levels. From those researches, Fe content ranges from 841.01 to 1820 mg/kg which is much higher compared with Fe content in barley genotypes examined in our paper. According to Shar et al. (2013), the content of other elements was as follows: Zn from 30.24 mg/kg to 53.05 mg/kg, Mn from 21.93 mg/kg to 38.69 mg/kg, Cu from 7.02 mg/kg to 10.46 mg/kg, Ni from 0.56 mg/kg to 1.23 mg/kg, and Al content from 10.42 t mg/kg to 25.38 mg/kg. Those concentrations were much higher compared to results obtained from our investigation except for nickel.

Šterna et al. (2015) also analyzed macro- and microcontent in four barley varieties. According to this investigation, Mg content ranges from 1123 to 1210 mg/kg, Ca from 309 to 353 mg/kg, Cu from 2.00 to 3.42 mg/kg, Mn from 11.80 to 16.37 mg/kg, Fe from 31.70 to 45.07 mg/kg, and Zn from 17.60 to 23.80 mg/kg. The results for macro- and microelements from our study differ more compared to results obtained in Šterna et al.'s (2015) research.

In many researches multivariate analyses such as principal component analysis (PCA), linear correlation (LC), and cluster analysis (CA) are necessary for proper examination and analysis of agro-morphological and chemical properties. According to Mohammadi and Prasanna (2003), most frequently used approaches are principal component analysis and cluster analysis, which are used for estimation of genetic diversity in various crops such as wheat (Hailu et al. 2006), sorghum (Ayana and Becele 1999), corn (Kamara et al. 2003), sunflower (Kholghi et al. 2011; Markova Ruzdik et al. 2015), and barley (Žàkovà and Benkovà 2006; Markova Ruzdik et al. 2016). PCA has been widely used in plant science, and the objective of this analysis is reduction of dimensionality of a data set with a large number of correlated variables of traits (Jolliffe 2002).

In this study, principal component analysis was utilized to examine the variation and to estimate the relative contribution of tested elements for total variability. Principal component analysis was carried out by using 30 barley winter genotypes and 10 elements. Two main components were extracted with Eigen value greater than one (Table 10.12). The result showed that 70.591% of the variability was explained by two main components. The first component explained 51.295% of the total variation, and the second PC component had 19.295% of total variance. Manganese and phosphorus had highly positive contribution to the first main component (Table 10.12). On the other side, the content of sodium, aluminum, and calcium showed negative factor loading in PC2 (−0.587, −0.524, and −0.402, respectively). The second main component was positively linked with cooper value (0.325).

Factor loading of tested barley genotypes by main components is summarized in Table 10.13. From all tested varieties, the following genotypes showed positive values among both main components: Hit, Line 1, Line 2, NS 525, Obzor, Emon, Orfej, Kuber, Sajra, Odisej, NS 589, Petra, and GK Judy. The positive factor loading by both main components indicates that the most suitable for breeding, according to the tested elements, were those varieties.

Table 10.12 Principal component analysis and factor loading of tested elements of barley genotypes

Parameter	PC1	PC2
Eigen values	5.123	1.930
Percent of variance	51.295	19.295
Cumulative percentage	51.295	70.591
	Factor loading of tested elements	
Na	0.156	−0.587
Mg	0.415	0.003
Al	0.213	−0.524
P	0.407	0.111
Ca	0.156	−0.402
Mn	0.339	0.182
Fe	0.395	−0.132
Cu	0.352	0.325
Zn	0.370	0.197
Ni	0.189	0.117

Table 10.13 Factor loading of tested barley genotypes by main components

Genotype	PC1	PC2
Hit	2.244	1.108
Izvor	1.054	−2.395
Egej	0.186	−0.201
Line 1	0.094	0.744
Line 2	2.086	1.366
Zlatko	0.196	−0.196
Rex	−0.193	0.039
NS 525	2.571	1.196
NS 565	−0.460	0.831
Obzor	1.661	0.200
Perun	−0.150	0.670
Emon	0.966	1.145
Lardeya	−0.977	1.308
Orfej	1.364	0.812
Imeon	0.314	−0.389
Zagorec	0.470	−0.068
Asparuh	−1.552	0.747
Kuber	1.323	0.561
Sajra	0.986	0.593
Devinija	−3.084	0.116
Odisej	2.887	1.077
NS 589	3.900	0.881
Somborac	−1.875	−0.628
Javor	−1.510	−0.820
Kompolti korai	−3.271	−0.469
Petra	2.995	0.831
GK Stramm	−2.716	−0.367
GK Judy	0.738	0.994
Liliana	−4.669	−0.665
Univers	−5.576	−0.141

In order to determine similarity or distance between tested barley genotypes based on macro- and microcontent, cluster analysis has been performed (Fig. 10.4). The dendrogram illustrates that based on macro- and microcontent, barley genotypes are clearly grouped in four clusters. The first cluster is the smallest and is consisted of three varieties (Hit, Izvor, and Petra). The similarity between Hit and Petra varieties is based on zinc content. The second cluster is the biggest one and contains 16 genotypes. Within the frame of this cluster, genotypes Line 1 and NS 565 are most similar; they are linked in a subgroup and have least distant unit (difference). Also, the least distant unit was obtained between the following genotypes: Rex and Perun, Imeon and Zagorec (Bulgarian two-row varieties), Line 2 and Sajra, Kuber and GK Judy, and Emon and Orfej (two-row cultivars with Bulgarian origin).

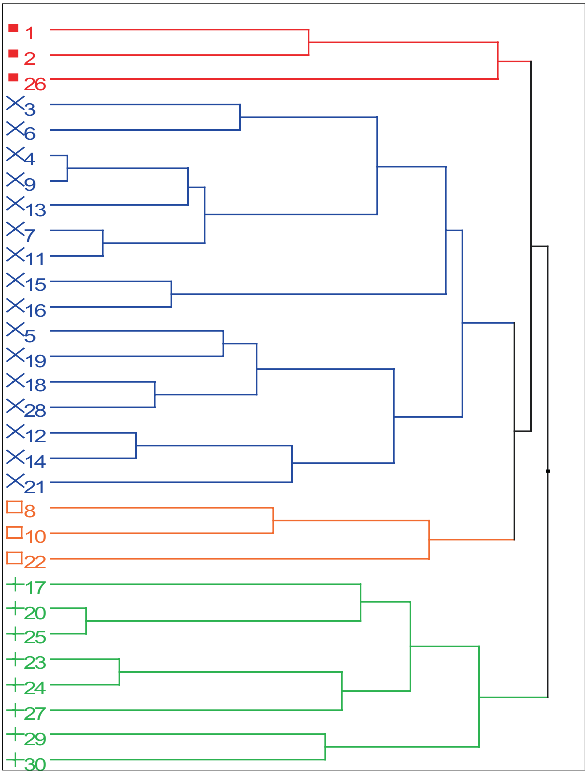

Fig. 10.4 Hierarchical cluster analysis of elements in barley genotypes (Ward method). (Legend: 1. Hit, 2. Izvor, 3. Egej, 4. Line 1, 5. Line 2, 6. Zlatko, 7. Rex, 8. NS 525, 9. NS 565, 10. Obzor, 11. Perun, 12. Emon, 13. Lardeya, 14. Orfej, 15. Imeon, 16. Zagorec, 17.Asparuh, 18. Kuber, 19. Sajra, 20. Devinija, 21. Odisej, 22. NS 589, 23. Somborac, 24. Javor, 25. Kompolti korai, 26. Petra, 27. GK Stramm, 28. GK Judy, 29. Liliana, 30. Univers)

The third cluster is composed of the following varieties: NS 525, Obzor, and NS 589. NS 525 and NS 589 are Serbian varieties with two-row spike morphology and belong in the same cluster because they have the highest values for Na, P, Cu, Zn, and Ni. The fourth cluster is consisted of eight cultivars. All six-row barley genotypes belong to this cluster, except Petra. Within the cluster there are two subgroups. The first one was between Devinija and Kompolti korai, while the second was between genotypes Somborac and Javor, which have six-row spike and Serbian origin.

10.4 Conclusion

This work aimed to study the macro- and microcontent in 30 winter barley varieties with different origin, grown in the same environmental conditions, and to evaluate the differences in mineral content in two- and six-row barley genotypes. The data presented in this paper demonstrated considerable variations in macro- and microcontent in barley genotypes cultivated in the Republic of North Macedonia. From the results it can be marked that two-row barley genotypes contained significantly larger amount of tested elements compared to varieties which have six-row spike morphology, except for calcium. From the other side, Bulgarian varieties had lower calcium content compared to other two-row varieties. NS 525 variety showed the highest levels of sodium, cooper, and nickel, while the highest content of phosphorus and zinc was obtained by NS 589 cultivar. Macedonian genotype Line 2 had the highest concentration of manganese, and in two-row barley variety, Hit established the largest content of aluminum and iron. Bulgarian variety Odisej showed the highest content of manganese and high values for zinc and cooper. From all tested varieties, in Univers cultivar was determined the smallest content of manganese, phosphorus, iron, zinc, and nickel. PCA analysis showed that 70.591% of the variability was explained by two main components. The first component explained 51.295% of the total variation, and the second PC component had 19.295% of total variance. The positive factor loading by both main components had the following genotypes: Hit, Line 1, Line 2, NS 525, Obzor, Emon, Orfej, Kuber, Sajra, Odisej, NS 589, Petra, and GK Judy. From the other side, the genotypes NS 525 and NS 589 belong to the same cluster group. These differences in grain chemical content may be utilized in barley breeding programs particularly to produce varieties for specific purpose, also for feed. From all tested genotypes, a few of them (NS 525, NS 589, Hit, Line 2, and Odisej) can be used in barley breeding programs, like parents in order to improve chemical composition in barley and also the nutritional grain quality.

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Chapter 11
Chemical Composition and Nutritional Properties of Functional Food

Violeta Ivanova Petropulos and Biljana Balabanova

Abstract Functional foods and beverages provide an extra health benefit to the consumer beyond basic nutrition. Examples include foods that have been fortified with beneficial nutrients or foods that are free from certain components such as lactose or gluten-free. Also known as nutraceuticals, functional foods are highly nutritious and associated with a number of powerful health benefits, positive effect on certain functions in the body, in addition to its usual nutritional effect, in terms of promoting health and reducing the risk of disease, preventing nutrient deficiencies, and promoting proper growth and development. Most of the functional food's products have functional properties due to the presence of one or more components (biologically active compounds), with favorable physiological effects. A biologically active compound can be a macronutrient (resistant starch or n-3 fatty acid), a micronutrient (vitamin or mineral), and a nonessential food ingredient that has a certain energy value (oligosaccharides, conjugated linoleic acid, plant sterol, lycopene). Various chemical methods are used to monitor food quality, such as liquid chromatography, gas chromatography, and atomic spectrometry, connected with various detectors. The main reasons why chemical analyses are very important today are time-savings, often greater accuracy in work, the ability to analyze very small samples, and the ability to avoid often very complicated chemical separations.

Keywords Functional food · Chemical composition · Nutrients · Analytical techniques

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11.1 Introduction

Until relatively recently, people got their food directly from nature. It is only in the last hundred years that technology has taken the lead and we have developed an unlimited number of food products from the original foods. The modern concept of functional food is thought to have been conceived in Japan 25 years ago, but the understanding of food as a medicine is certainly not of recent date. The father of medicine, Hippocrates, put this hypothesis back 2500 years ago, and ancient Eastern civilizations traditionally associate certain foods with specific effects on health. Yet the philosophy of "food as medicine" was unfairly neglected during the nineteenth century due to the dawn of modern drug therapy. The important role of nutrition in disease prevention and health promotion comes to the fore again in the early twentieth century. This era was marked by many revolutionary discoveries, such as the discovery of vitamins and numerous diseases that arose as a result of deficiencies in certain nutrients. Nutrients added to a staple food can increase the intake of a particular nutrient even at the level of the entire population (Devcich et al. 2007). It was the enrichment of food with key nutrients in the early twentieth century that was responsible for eradicating diseases such as goiter, rickets, beriberi, and pellagra. In the 1970s, the focus of interest shifted sharply from nutrient deficiencies to overweight. The beginnings of a "society of abundance" that has its roots in that age are being felt in full swing today. The numbers speak for themselves – an average of 30–50% of the Western population is overweight, and all indications are that we are heading toward a global obesity epidemic (Benkouider 2004). During the 1970s and 1980s, a whole range of physiologically active substances of plant and animal origin (phytochemicals and zoochemicals) were identified, with potentially beneficial effects on the prevention and treatment of chronic diseases (Roberfroid 2002; Niva 2007).

Today, people are increasingly consuming food, counting on its ingredients, which are found in small quantities and which can have a beneficial effect on health (Devcich et al. 2007). Such ingredients are called biologically active components of food. The quality of these substances is not measured by their quantitative presence but by the level of ability to neutralize the effects of harmful substances in the human body during their biological activity. All biologically active components differ from each other both in chemical structure and in function in the organism. Some of these ingredients include vitamins and minerals, carotenoids, phenolic compounds, flavonoids, phytosterols, isoflavones, enzymes, tannins, glycosides, alkaloids, and others. All these substances, ingested in adequate quantities, can have a beneficial effect on health and on various systems in the body such as gastrointestinal, cardiovascular, endocrine, nervous, immune. and others (Devcich et al. 2007).

On the other hand, these food ingredients can influence the course of prevention and treatment of many diseases, such as modern diseases of the metabolic syndrome, cancer, or diseases caused by nutrient deficiency or deficiency. Thus, foods that contain protective factors (milk, probiotics, whey, white chicken, fish meat and omega-3 fatty acids, liver, dark green and yellow vegetables, citrus fruits, whole grain bread, etc.) are recognized today. Knowing the choice of individual foods based on ingredients that are recommended and not allowed for certain people allows, for example, the formulation of food for various metabolic disorders (Roberfroid 2002; Marinangeli and Jones 2010). Some foods can be excluded only in certain metabolic disorders (phenylketonuria, gluten enteropathy, lactose and fructose intolerance, etc.). For example, in phenylketonuria, everything is given except phenylalanine, which the body cannot accept due to the disorder (Marinangeli and Jones 2010). It is considered that bioactive substances are mostly found in plants (fruits, vegetables, cereals). Some of them, such as probiotics, conjugated linolenic acid, omega-3 fatty acids, and bioactive peptides, are found in animal products, such as milk, dairy products, and fish. The application of diet therapy and nutritional modifications in many diseases is crucial for the course and outcome of the disease. In recent decades, a large number of specially designed food products and food supplements have appeared on the market, which are becoming increasingly popular (Maynard and Franklin 2003). The interest in such food, which is in fact somewhere on the border between food and medicine, is huge, due to the better understanding of the connection between diet, health, and disease (Marinangeli and Jones 2010). The diversity of food types in terms of nutritional attributes classifies food into various categories such as: therefore diet food, food for pregnant and lactating women, baby food, food for the elderly, food for menopausal women, food for managers, food for athletes, but also various dietary supplements and functional foods, etc. In addition to nutrients, these types of foods also require the presence of biologically active components (Marinangeli and Jones 2010).

11.2 General Aspects for Functional Food

There are many definitions of functional foods. Simply put, functional food, in addition to its energy function, also has a role in maintaining health. This means that it contains food ingredients that are known to have a positive effect on human health, reduce the risk of developing the disease, or can even be used during treatment to help the body recover faster. It does not have to have an impact on the entire population. Functional food should have the following characteristics:

- To be consumed in the daily diet.
- That it is a conventional food and not "exotic."
- It should have a natural composition, without chemical additives.
- If certain components are added, they should only supplement the naturally occurring amounts.
- In order to justify the name, it should have a positive effect on physiological functions and can reduce the risk of developing the disease.
- Health claims attributed to these foods need to be confirmed and substantiated.

There is a whole spectrum of different chemical compounds that have certain effects on human health or certain physiological functions. These compounds are being researched more and more every day, and by their action, they are not medicines, but they can sometimes be isolated and used as medicine (Niva 2007). The situation is similar with herbal preparations. According to their origin, nutraceuticals can be herbal, animal, and microbiological. Of plant origin are all plant pigments, beta-glucan, plant acids, MUFA (monounsaturated fatty acids), etc. In the animal world, the following are interesting: polyunsaturated fatty acids, sphingolipids, choline, lecithin, calcium, coenzyme Q10, selenium, zinc, creatine, etc. The most talked about microorganisms are yeast (*Saccharomyces boulardii*) and probiotic bacteria (*Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Streptococcus salivarius*, etc.). This division is not solid because many of them can be found in several groups at the same time. Thus, we distinguish between antioxidants, immunomodulators, antipyretics, anti-dyslipidemic substances, etc. Every day the applications grow and the groups of action expand. The largest group of functional foods is unmodified and unprocessed foods: fruits, vegetables, and herbs. Many fruits and vegetables contain pigments and phytochemicals that are known to have beneficial effects on health. The situation is similar with herbs (Chiorcea-Paquim et al. 2020).

The concept of "self-medication" and caring for one's own health through specially designed food products and food supplements is becoming increasingly popular. The market for nutraceuticals, supplements, and all other products that are somewhere on the border of food and medicine is growing day by day. It is difficult to draw firm boundaries between conventional, functional foods, organically grown foods, supplements, and nutraceuticals, and even scientists find it difficult to handle these terms. Therefore, the broad concept of functional food can include naturally nutritious food, food enriched with functional ingredients, food from which certain substances have been removed, food in which the properties of individual components have been changed, food in which the bioavailability of one or more components has been modified, and all combinations of the mentioned possibilities.

Functional food is nothing but traditional food that contains additives that make it functional (Benkouider 2004). These foods can be categorized according to the effect on the health of an individual organ or organ system, such as the health of the digestive system, skeletal system, blood vessels, or immune system. The best and most widespread example in our country is good old yoghurt, which becomes functional with the addition of probiotic bacteria. Probiotics or "beneficial bacteria" with their metabolism create unfavorable conditions for the growth of pathogenic microorganisms, help us get all the important nutrients and energy from food, and are also responsible for the synthesis of vitamin K and some B vitamins. A favorable balance of intestinal microflora is thought to play an important role in developing and maintaining a strong immune system. Calcium and vitamin D are added to yogurt, milk, and fruit juices to reduce the risk of osteoporosis. Regular exercise and a proper diet with enough calcium and vitamin D helps adolescents and young adults maintain good bone health and reduce the risk of osteoporosis later in life. Vitamins and minerals are added to a whole range of products, especially cereals and fruit juices. Vitamins A, C, and E due to their antioxidant action play an important role in preserving the immune system and defending against malignant diseases

(Maynard and Franklin 2003). Folic acid, a vitamin of the B group, has found its place as a supplement to many foods due to a revolutionary discovery about the role of this nutrient in newborn health. With adequate folic acid intake before and in the first months of pregnancy, a woman greatly reduces the likelihood of having a baby with a neural tube or brain damage (Weststrate et al. 2002). Products designed to preserve the health of the cardiovascular system most often contain the addition of omega-3 fatty acids, soy, and the phytochemicals phytostanol and phytosterols. These food-derived substances are thought to have a beneficial effect on blood cholesterol levels. Demand for functional foods is growing due to a better understanding of the link between diet and disease or health and an individual's growing interest in their own health (Wansink et al. 2005; Devcich et al. 2007).

11.3 Biologically Active Ingredients of Food

Food consists of a large variety of known and unknown natural biologically active ingredients, which can contribute to improved health. Biologically active ingredients in food are most commonly defined as natural nonnutritive food ingredients that have beneficial effects on health if consumed in adequate amounts. The term biological activity can also refer to harmful effects on health, and then we talk about toxicity of these ingredients. To understand the influence of biologically active components from food, one should keep in mind Paracelsus' statement: "All substances are poison and nothing is poison, only the dose determines something that will not be toxic." Consumption of a certain food ingredient can have detrimental effects on health, especially those that have higher biological activity in metabolic processes in the human body. Therefore, for most nutrients, there are recommended (RDA) and reference (DRI) daily intakes, as well as maximum intake limits (MIL). That is why the amount is important for the biologically active ingredient of food, because excessive amounts can create the opposite effect than desired (WHO 2003). Therefore, dietary aspects should also be considered, as part of lifestyle, in the process of assessing the need for food intake that contains certain biologically active components (Marinangeli and Jones 2010).

The term biologically active food ingredient most often refers to compounds that have no nutritional value but have functional properties in the body and act as adjuvants in the prevention and treatment of disease. Numerous plant species contain substances that have an impact on the health of the organism. Thus, for example, the most frequently mentioned nonnutritive biologically active components are sulforaphane, indole, and carbinol in broccoli; lycopene in tomatoes and watermelons; beta-carotene in carrots, apricots, and peaches; lutein in corn; allicin in garlic; quercetin in onion; ellagic acid in apples; genistein in soy; capsaicin and carotenoids in hot peppers; and catechins in green tea. These compounds are not classified as nutrients, but they play a significant role in the diet, precisely because of their pronounced protective function. There are no RDA and DRI recommendations for these compounds yet, but the importance of their consumption has been pointed out in numerous dietary guides and diets (WHO 2003). Many nutrients, such as omega fatty acids, essential fatty acids, many amino acids, vitamins, and minerals, are known to have effects on their metabolic role in the prevention and treatment of certain clinical conditions (WHO 2003). However, many of the mechanisms through which nonnutritive and, to a large extent, nutritional bioactive components of food affect health have not yet been sufficiently investigated. Biologically active food ingredients are physiologically active constituents in food or dietary supplements derived from plant and animal sources and include nutritional ingredients that have been shown to play a useful role in disease prevention and health (WHO 2003). Clinical studies, as well as good dietary practice, show that specific components of food have the potential to reduce the risk of some diseases, such as diseases of the digestive system, metabolic syndrome, cancer, osteoporosis, inflammation, and other chronic diseases (WHO 2003; Devcich et al. 2007). Thus, in cardiovascular diseases, isoflavones can reduce the circulation of low-density lipoprotein in plasma; crude fiber can bind cholesterol in the gastrointestinal tract and thus reduce the absorption of cholesterol and thus the level of cholesterol in the blood. Of particular importance are antioxidants that eliminate the influence of free radicals in the body. Some foods have particularly high biological activity, such as colostrum and whey, pollen, propolis and royal jelly, omega-3-essential fatty acids, and others (Devcich et al. 2007).

Phytochemicals from fruits, vegetables, and herbs such as chlorophyll, carotenoids, polyphenols, flavonoids, glycosides, tannins, alkaloids, phytosterols, lignans, and others can have high biological activity. Biologically active food components can be found in any of the existing regulatory food categories, including conventional foods, dietary supplements, some food additives, medical foods, foods for special nutritional needs, or dietary food products.

11.3.1 Characterization of BAC (Biologically Active Components)

Biologically active components (BAC) of food can be divided according to several criteria: according to the chemical composition, according to the origin, according to the function of action on certain organs and tissues in the body, and according to the form or pharmaceutical form if consumed as food supplements. Biologically active food compounds can be of a nutritional nature, e.g., amino acids, fatty acids, some carbohydrates (e.g., beta-D-glucans, inositol, sorbitol), vitamins, and minerals, and nonnutritive origin such as polyphenols, flavonoids, chlorophyll, isothiocyanates, phytoestrogenic glycosides, alkaloids, and steroids.

Some of the biologically active components of food primarily act on the digestive system such as dietary fiber and enzymes in food (bromelain, papain, amylase); some act on other systems such as the vascular (omega-3 fatty acids, vitamin K, ginkgo biloba), endocrine (alpha lipoic acid), nervous system (xanthine derivatives), etc. According to the origin, biologically active components can be plant (carotenoids, polyphenols, flavonoids, betalains, glycosides, alkaloids, saponins), animal (colostrum, probiotics, whey proteins), mineral (Ca, Mg, I), and microorganisms (functional microbial cultures). The more industrially processed the food, the lower the proportion of phytochemicals. When the food is refined to the end, then no phytochemicals remain in it, such as white sugar, alcoholic vinegar, or spirits. The simplest way to ensure the presence of these health-beneficial compounds is to have a dietary diversity rule and to provide 5–10 servings of fruits and vegetables daily. This ensures the intake of other nutrients important for the functioning of the body (vitamins, minerals, fiber, essential fats) and then phytochemicals. As food ingredients in their natural form, biologically active components can be in different physical states, often composed of numerous active chemical ingredients (colostrum, probiotics, whey proteins). If they are used as food supplements, then according to the consistency, they can be in solid, gelatinous, and liquid state, and according to the pharmaceutical form, they can be in the form of tablets, capsules, holders, powders, syrups, etc. (WHO 2003).

11.3.2 The Effect of BAC on Certain Systems in Humans

There are many types of food that can affect different systems in the human body, and some food ingredients are almost universal, such as antioxidants: vitamin C and vitamin E, selenium, zinc, polyphenols, flavonoids, carotenoids, colostrum, royal jelly, etc. Research on phytochemicals and their impact on health has been gaining increasing attention in recent decades (Marinangeli and Jones 2010). Phytochemicals are a group of biologically active nonnutritive substances from plants. They have a functional value for the human body, acting in terms of protection against disease or prevention. For example, they can act on the immune system immunomodulator, adaptogen to the endocrine system, and protective bone system and be antimutagenic and anticarcinogenic at the cellular level, etc. Areas where their effects are currently being intensively investigated are cardiovascular diseases, carcinomas, bone diseases, digestive and endocrine system health, immunity, and chronic degenerative diseases (Devcich et al. 2007). Since the organism is a complex system where the functions of individual organs are very closely related, the biologically active components will also have an impact on the overall condition of the organism. For example, the action of antioxidants to reduce oxidative stress affects the overall defense system of the body; the action of crude fiber in the digestive system improves peristalsis and absorption of nutrients, which affects the proper supply of nutrients to the bloodstream, etc. It is best to show this by the example of vitamin C which is involved in more than 300 biological processes in the body (Shahidi 2009). Some of them are collagen biosynthesis, the hormonal system, circulatory system, nervous system, etc. Vitamin C is needed in vivo (in a living organism) as a cofactor for at least eight enzymes. The best known are proline hydroxylase and lysine hydroxylase which participate in collagen biosynthesis. Both enzymes contain iron in their active sites. Collagen synthesized in the absence of vitamin C is insufficiently hydroxylated, so the fibers are not firm, which causes the fragility of blood vessels. Collagen is a basic connective tissue protein, necessary for the construction and protection of blood vessels, bones, joints, and muscles. Vitamin C is involved in the synthesis of adrenal hormones. It is required for the synthesis of the enzyme dopamine hydroxylase, which contains copper and converts dopamine into norepinephrine. Vitamin C is important in the formation of cholesterol in the liver and its conversion into bile acids and stimulates normal cholesterol and LDL cholesterol levels in the blood. It also affects normal circulation, which is important for heart function and maintains normal blood pressure. Vitamin C is involved in the conversion of the amino acid tryptophan into serotonin, a transmitter of nerve stimuli with numerous functions of the nervous system. However, biologically active compounds, it can be freely said, have a priority effect in a certain system and indirectly act on others and improve the general condition of the organism (Marinangeli and Jones 2010).

11.4 Chemical Composition of Significant Biologically Active Food Components

The biologically active components of food are of very different chemical composition, and it is common to use their trivial names through which both the chemical composition and the biological activity are identified (Roberfroid 2002). These are most often secondary metabolites of plants and other organisms. They can be pigments that give food color (chlorophylls, flavonoids, hemoglobin), substances that protect plants from herbivores (polyphenols, terpenes, and other waxy substances), substances that give aroma, etc. Plant pigments give color to fresh foods as well as the products made from them. The most famous is chlorophyll in green plants and a large group of carotenoids and flavonoids. The green color of leafy vegetables comes from the chlorophyll that is responsible for photosynthesis. Yellow and orange colors are derived from carotenoids. Flavonoids and carotenoids are pigments in food mainly present in plant foods. These are compounds that have a lot of unsaturated bonds that break easily. Red, blue, and purple colors are derived from flavonoid compounds, especially anthocyanins, which are good antioxidants. The content of pigments is relatively low, but even in small concentrations they give color to the product. They can be soluble and insoluble in water. They are often bound to other substances within the cell. Plant pigments develop during ripening and maturation. We will give a few examples of biologically active components in food classified by characteristic chemical compounds or food components.

Vitamins Vitamins are diverse compounds with regard to structure, biological activities, and their chemical properties. Vitamins are essential in trace amounts for the normal growth and maintenance of life. To ensure the adequate intake of vitamins, the human diet can be completed with a high range of multivitamin tablets and food products enriched with vitamins, in other words, these compounds are usually administered as nutraceutical or functional ingredient. They are classified as either *water-soluble* or *fat-soluble* (Figs 11.1a, b). The water-soluble vitamin group includes thiamine and its derivatives, vitamin B_1, riboflavin (vitamin B_2), niacin (nicotinic acid) and its amide (nicotinamide, vitamin PP), vitamin B6 vitamers (pyridoxal, pyridoxine, and pyridoxamine and their derivatives), pantothenic acid, folic acid and its derivatives (folacin), biotin (vitamin H), and cyanocobalamin (vitamin B_{12}), in addition to L-ascorbic acid and L-dehydroascorbic acid (vitamin C) and other biologically active compounds, classified as pseudo-vitamins, such as orotic acid (vitamin B_{13}), 4-aminobenzoic acid, inositol (myo- and meso-inositol), rutin (vitamin P), and S-methylmethionine (vitamin U). The fat-soluble vitamins fall into four groups of vitamin-active compounds: vitamin A (retinoids and carotenoids), vitamin D [ergocalciferol (D_2) and cholecalciferol (D_3)], vitamin E (tocopherols and

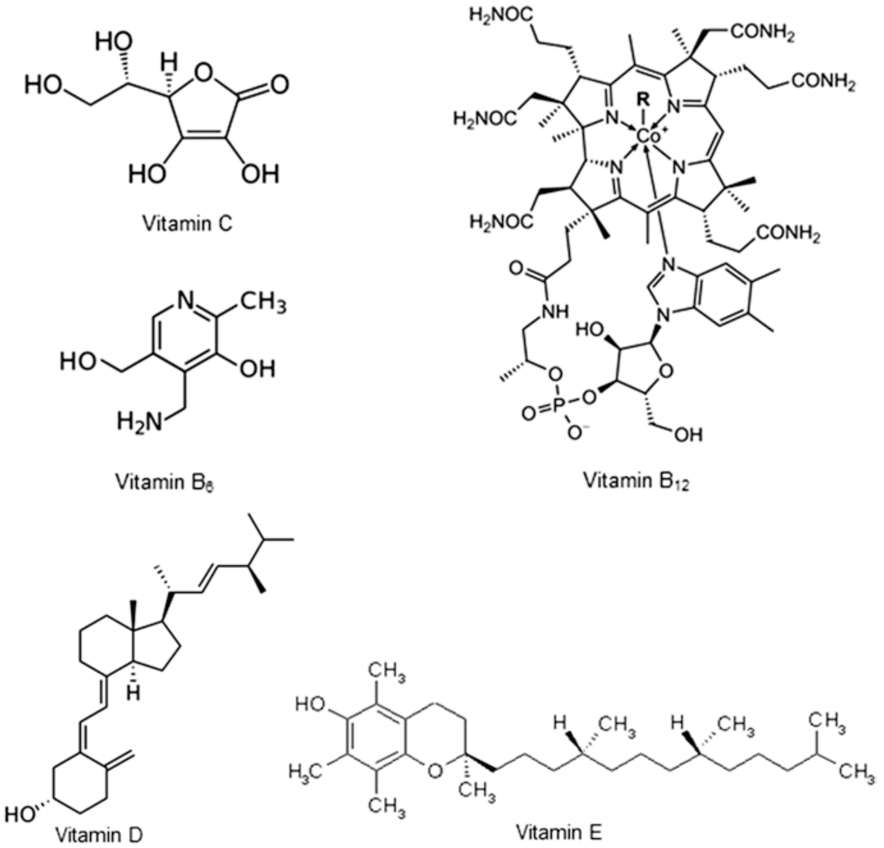

Fig. 11.1 (**a**) Structures of some water-soluble vitamins. (**b**) Structures of some fat-soluble vitamins

tocotrienols), and vitamin K (phylloquinone and menaquinones) (Rizzolo and Polesello 1992).

Some vitamins have hormonelike functions as regulators of mineral metabolism (e.g., vitamin D) or regulators of cell and tissue growth and differentiation (e.g., some forms of vitamin A). Others work as antioxidants (e.g., vitamin E and sometimes vitamins B and C). The largest numbers of vitamins (e.g., B complex vitamins) work as precursors of enzyme cofactors (Bernard et al. 2011). Vitamin C (L-ascorbic acid or L-ascorbate) is an essential nutrient for humans and other animal species. Deficiency in this vitamin causes the disease known as scurvy in humans. This compound is also widely used as a food additive because of its antioxidant activity. Vitamin E is a generic term for tocopherols and tocotrienols, and it is a fat-soluble antioxidant that blocks the production of reactive oxygen species formed when lipids undergo oxidation.

Carotenoids Several hundred compounds belonging to carotenoids are known today, synthesized by plants, algae, yeast, fungi, and photosynthetic bacteria. They are prominent for their distribution, structural diversity, and various functions. Fruits and vegetables provide most of the carotenoids in the human diet (Bernard et al. 2011). Carotenoids are soluble in oils and organic solvents, and their color varies from yellow through orange to red. Two structural groups of carotenoids are distinguished: carotenes (α-carotene, β-carotene, or lycopene) and xanthophylls (β-cryptoxanthin, lutein, or zeaxanthin). The basic structure of carotenoids consists of covalently linked isoprene units. At the end of each chain, there is either a ring or an open chain, and the carotenoids differ from each other. These are polyunsaturated compounds. The most common carotenoid in the plant world is β-carotene, and other carotenoids found in plants are α-carotene (carrots), capsanthin (red peppers, peppers), lutein, zeaxanthin, violaxanthin, neoxanthin, and β-cryptoxanthin. These compounds show antioxidant and immunomodulation activities, and they can prevent degenerative diseases, such as cardiovascular diseases, diabetes, and several types of cancer especially prostate and digestive tract tumors.

Polyphenols Polyphenols are secondary metabolites of plants in which they have multiple roles:

- Sensory properties such as color, aroma, or taste.
- Affect the plant's resistance to diseases and microorganisms.
- Some polyphenols indirectly affect plant growth as well.
- Protect sensitive cellular parts from harmful radiation (UV absorption).

Polyphenols include more than 10,000 compounds of various chemical structures which have been isolated and identified. The word "polyphenol" is formed from the ancient Greek word "poli-," which means "much," and the word "phenol" which is a molecule formed from a phenyl (-C_6H_5) group linked to a hydroxyl (-OH) group. They are essential for the growth and reproduction of plants and are produced to defend injured plants against pathogens. They are secondary metabolites of plants and are known as hormones, vitamins, and food antioxidants.

Polyphenols are divided according to their chemical structure, although they can also be classified by their source of origin, natural distribution, or biological function. In particular, according to their chemical structure, they can be classified into different groups, as function of the number of phenol rings contained and the structural elements that bind these rings. The phenol structure has one phenolic –OH group; catechol, hydroquinone, and resorcinol (benzenediol) structures have two phenolic –OH groups; and gallocatechol and phloroglucinol (benzenetriol) structures have three phenolic –OH groups. Most polyphenols are formed by the deamination of the phenylalanine and tyrosine amino acids to cinnamic acids, as part of the phenylpropanoid pathway. Although these compounds have a common carbon C6-C3 phenylpropanoid element, an essential step in their biosynthesis is the attachment of one or more –OH groups to the phenyl ring, which gives rise to a great diversity of molecules: cinnamic acids (C6-C3), benzoic acids (C6-C1), flavonoids (C6-C3-C6), proanthocyanidins [(C6-C3-C6)n], coumarins (C6-C3), stilbenes (C6-C2-C6), lignans (C6-C3-C3-C6), and lignins [(C6-C3)n] (Chiorcea-Paquim et al. 2020).

In general, polyphenols are divided into two groups: *flavonoids* and *nonflavonoids*. The flavonoids consist of two benzene rings (A and B) linked by an oxygen-containing pyran ring (Fig. 11.2).

Flavonoids may be divided into following groups (Fig. 11.3):

The color of anthocyanins is directly linked to pH of the medium, whereas several studies have shown the presence of colored and uncolored forms of anthocyanins depending on the pH (Somers 1971) (Fig. 11.4).

The anthocyanidin system undergoes a variety of molecular transformations as the pH changes Thus, in aqueous solution, a mixture of several structures of anthocyanins exists in equilibrium: flavylium cation (red), quinonoidal anhydrobase (blue), carbinol pseudobase (colorless), and chalcone (colorless or light yellow). In highly acidic media, flavylium cations are mainly present, losing their color as the pH increases by formation of colorless carbinol bases, which in turn are in equilibrium with the open ring chalcone yellow form. The equilibrium between the carbinol and chalcone forms is very slow at room temperature and in slightly acidic media, while increased temperature displaces the equilibrium toward the chalcone forms. In aqueous media, the hydration of the flavylium cation gives the hemiacetal form in equilibrium with a chalcone. In neutral and alkaline medium, the equilib-

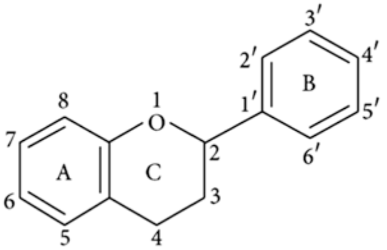

Fig. 11.2 Flavonoid structure

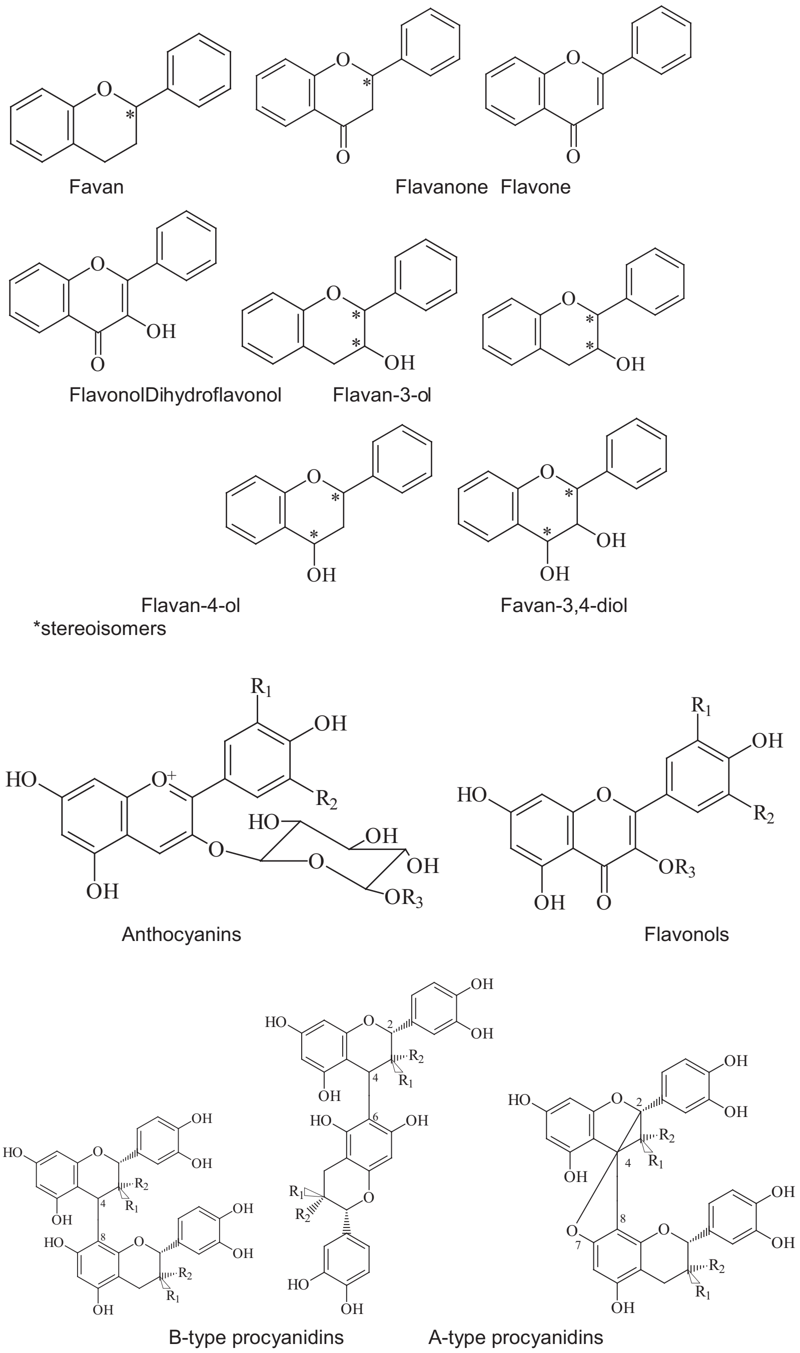

Fig. 11.3 Structures of various flavonoid groups

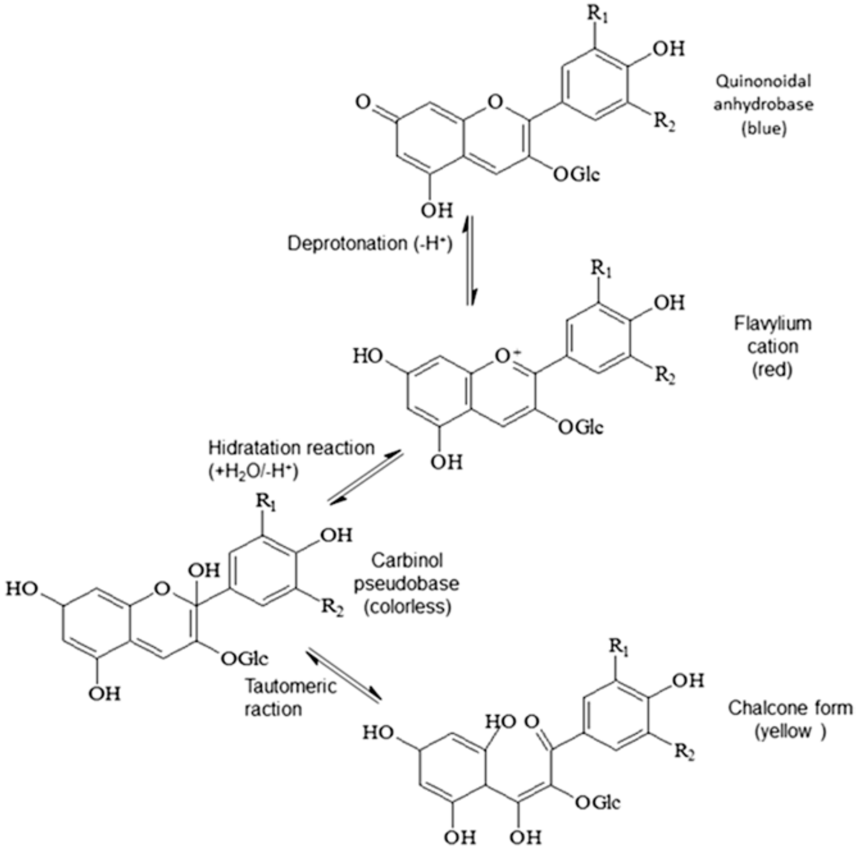

Fig. 11.4 Anthocyanin's transformation depending on the pH of the medium

rium is shifted toward a purple quinoidal anhydrobase which is responsible for the perceived color.

Nonflavonoid group consists of hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and volatile phenols (Fig. 11.5).

Polyphenols are soluble in water. They have a molecular weight of 500–4000 Da. They are composed of 12–16 phenolic groups with 5–7 aromatic rings. They give the usual phenolic reactions such as the formation of an intense blue-black complex on treatment with iron(III) salts. They have pronounced special properties such as the ability to precipitate alkaloids and proteins. Many polyphenols are found in the form of glycosides. Olive oil, for example, is a tyrosol ester with fatty acids. Phenolic acids and flavonoids are significant active components in the structure of polyphenols. Phenolic acids are found exclusively in the outer layers of plants, especially in cereals and cabbage. They enter into a covalent bond with polycyclic aromatic carbohydrates and thus suppress their carcinogenic effects. In animal experiments, phenolic acids block the chemically induced development of cancer of

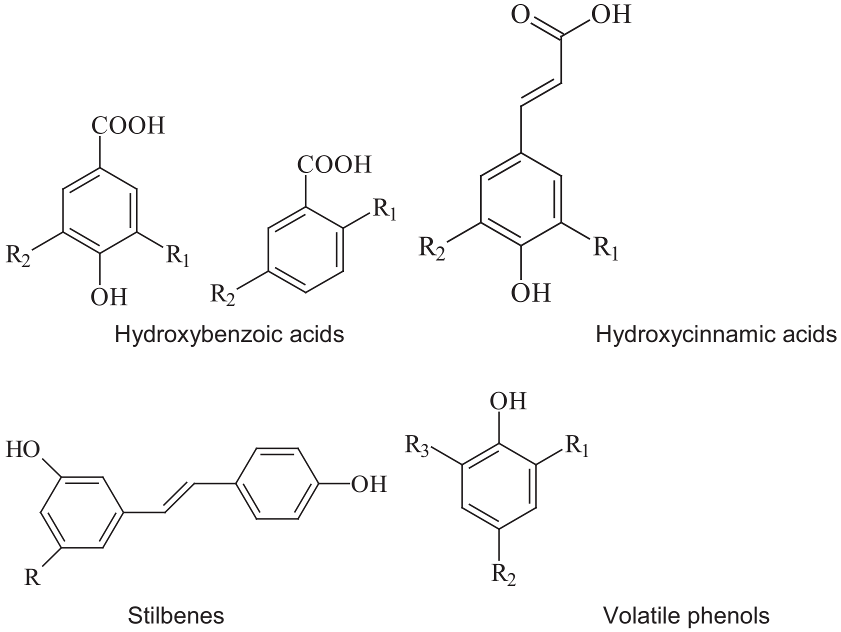

Fig. 11.5 Structures of nonflavonoid groups

the esophagus, stomach, skin, and lungs. Polyphenols include the aforementioned flavonoids, catechins, as well as resveratrol and anthocyanidins. The health effect of polyphenols depends on the amount consumed and their bioavailability. They have antioxidant properties: they are present in plants as secondary metabolites and are involved in protection against ultraviolet radiation and protection against pathogens. They are present in many medicinal plants and in food especially in tea, red wine, coffee, vegetables, legumes, as well as cereals.

Isothiocyanates, glucosinolates, free sterols, and sterol esters Isothiocyanates are compounds with the chemical group -N=C=S, which is formed by replacing sulfur with oxygen in the isocyanate group. Many natural isothiocyanates from plants are formed by enzymatic conversion from glucosinolates (organic compounds that contain sulfur and nitrogen and are derived from glucose and amino acids). Plants use substances derived from glucosinolates as a natural defense against herbivores. These substances are also responsible for the bitter and pungent taste of foods such as mustard, radishes, horseradish, cabbage, kale, kohlrabi, kale, cauliflower, broccoli, and beets. Glucosinolates are thioglycosides found in many plants (cabbage, cruciferous, mustard, radish, mustard, etc.). By enzymatic or chemical hydrolysis, they give acyclic and heterocyclic volatile compounds with N and/or S, which give these plants a pungent taste and odor. About 80 different glucosinolates are known in nature. The anticancer activity of glucosinolates and their metabolites of isothiocyanates, thiocyanates and indoles rest in the blockade of enzymatic processes of activation of procarcinogens into carcinogens and the induction of the so-called phase-II enzymes: e.g., glutathione-S-transferases, which accelerate the inactivation of activated carcinogens. Both animal experiments and clinical studies prove the influence of indole on estrogen metabolism, which thus exhibits a protective effect in estrogen-dependent tumors. Isocyanates from broccoli and other cabbages (kale, Brussels sprouts, cauliflower, cabbage) stimulate protective enzymes of the so-called second phase in the process of carcinogenesis, thus reducing the risk of cancer in various locations, because it is very rich in antioxidants and contains vitamin C and other flavonoids, including indoles and sulforaphane. Indoles prevent tumor growth, especially in the breast, and sulforaphane stimulates the production of cancer-fighting enzymes.

Alkaloids Alkaloids are present in a variety of food sources and are most commonly found in fruits, vegetables, spices, and aromatic herbs and are often used from medicinal plants. They can be classified into three groups: *protoalkaloids* (biogenic amines, ephedrine), *pseudoalkaloids* (steroidal and diterpene alkaloids), and *true alkaloids*. From the group of protoalkaloids, biogenic amines such as histamine are often present in small amounts in beer, wine, and fish. Biogenic amines are organic nitrogen compounds with low molecular weight who at high levels in wine can lead to health problems. The most important biogenic amines in wine are tryptamine, histamine, phenylethylamine, tyramine, putrescine, cadaverine, spermine, and spermidine (Fig. 11.6). The health risks arising from the presence of biogenic amines are related to symptoms such as nausea, respiratory discomfort, hot flushes, cold sweat, palpitations, headaches, red rash, high or low blood pressure, intracerebral hemorrhage, or even death. The most problematic biogenic amine is histamine, which causes headaches, low blood pressure, heart palpitations, edema, vomiting, and diarrhea (Tašev et al. 2016).

From the group of pseudoalkaloids are steroid and diterpene alkaloids that can also be found in various types of fruits and vegetables, most often in the cuticle, and

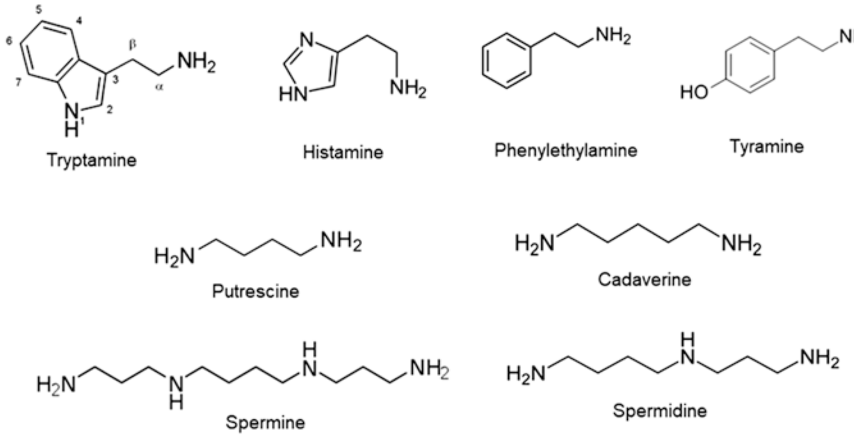

Fig. 11.6 Structures of biogenic amines

serve as protection for plants but are often also very biologically active. Caffeine, theobromine, theophylline, theine, guarana, capsaicin, piperine, and others are mostly used in the daily diet (Fig. 11.7). In addition to the nutritional effect on digestion and the body, alkaloids have a pharmacological effect. They have no energy value and are metabolized similarly to drugs. Of the alkaloids that have found their daily use in the diet, the most common are representatives of xanthine derivatives, capsaicin, piperine, and other alkaloids that are present in foods in small quantities.

Glycosides Glycosides or heterosides are conjugated organic compounds composed of a sugar glycone and a non-sugar component or aglycone (genin). The most

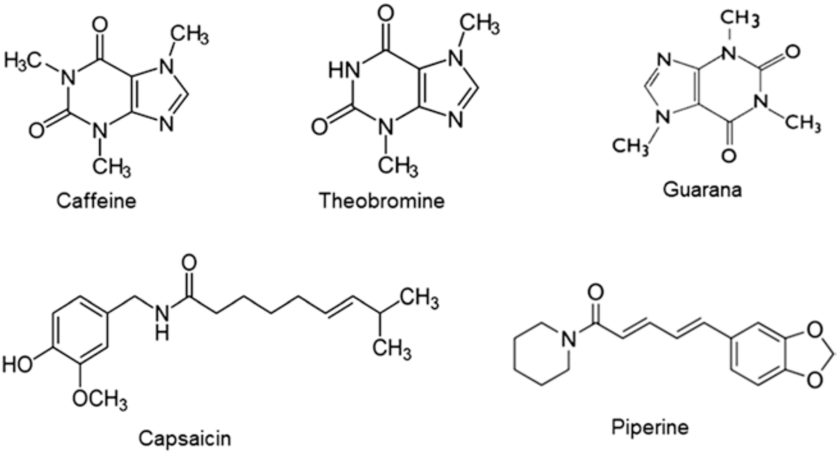

Fig. 11.7 Structures of pseudoalkaloids

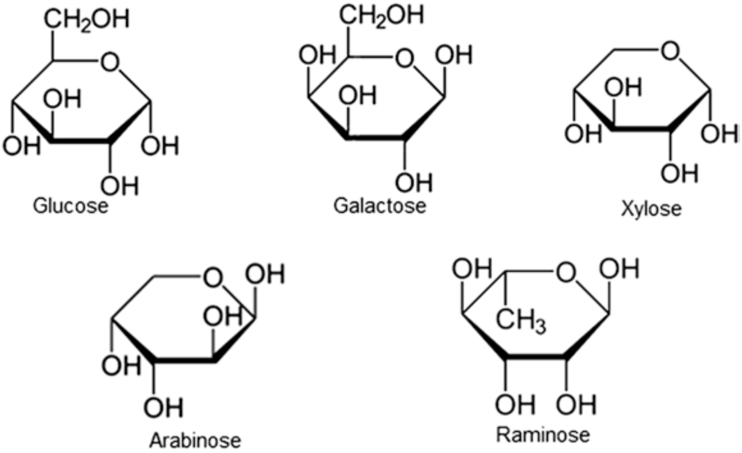

Fig. 11.8 Structures of glycosidic sugars

common glycosidic sugars are glucose, galactose, xylose, arabinose, and rhamnose (Fig. 11.8).

According to the type of sugar, glycosides can be called glucosides, rhamnosides, arabinosides, etc. If the glycoside contains several sugar molecules, they are interconnected into an oligosaccharide molecule. According to the number of sugar molecules, monoglycosides, diglycosides, triglycosides, etc. differ. Aglycones or genes are chemically very heterogeneous compounds. They can be alcohols (coniferyl), acids (benzoic), aldehydes (salicylic aldehyde), coumarins, xanthones, flavonoids, anthocyanin derivatives, anthraquinone derivatives, and cyclopentanoperhydrophenanthrene derivatives. Sugar is most often bound via O, but it can also be bound via C (thioglycosides), via N (nitrogen glycosides), etc. Glycosides are very common in plants. Many plant pigments are glycosides. Some plant families are especially rich in glycosides. Glycosides are important in pharmacy, as pharmacologically active substances, while in food they are found only in traces. Glycosides are found in smaller amounts in the meat of fruits and vegetables. They are present in the seeds as amygdalin and limetin, as well as in the epidermis (auranciamarin).

Fatty acids Depending on the number of hydrogen atoms attached to the free valences of carbon in the acid molecule, there are three types of fatty acids: *saturated*, *monounsaturated*, and *polyunsaturated*. Depending on the length of the fatty acid chain, they are divided into *short chain*, *medium chain*, and *long chain*. The maximum number of hydrogen atoms is attached to all carbon atoms in saturated fatty acids, i.e., all carbon atoms are "saturated" with hydrogen. This type of fatty acids dominates in fats that are solid at room temperature (fats of animal origin). Monounsaturated fatty acids (MUFAs) have such a chemical composition that allows them to bind two more hydrogen atoms in the fatty acid molecule. Fats containing such fatty acids are in a liquid state at room temperature. These foods are most often of plant origin and we call them vegetable oils. However, some fats are often called oil even though they are in a solid state at room temperature as is the case with palm oil. The most common fatty acid that belongs to the family of monounsaturated fatty acids is oleic acid (Fig. 11.9), the main ingredient in olive oil. The longer the acid residues, the higher the melting point. If the acids are unsaturated, the melting point with the number of double bonds decreases. Animal fats contain a significant amount of saturated fatty acids, especially stearic and palmitic. Animal fats are mostly solid at room temperature except for North Sea fish oil. If there are a lot of unsaturated acids in the molecule, then these are oils, which are liquid at room temperature (olive oil, soybean, sunflower, rapeseed oil).

The human body needs a full range of unsaturated fatty acids for its normal functioning, because they are an integral part of the body (cell membranes, various hormones, and enzymes). Most of the unsaturated fatty acids are created by the body itself, but it needs raw materials. These raw materials are actually essential fatty acids, which are necessary to enter the body from the outside through food and without which life is not possible. The human body needs only two essential fatty acids: linoleic (LA) from the group of omega-6 fatty acids and alpha linolenic

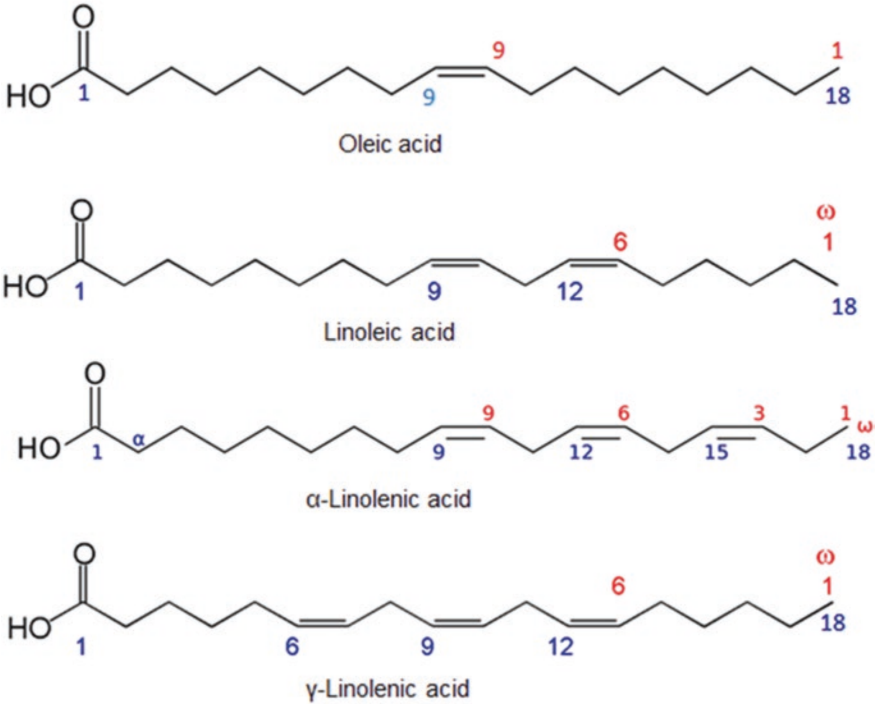

Fig. 11.9 Structures of fatty acids

(ALA) from the group of omega-3 fatty acids. Fatty acids with several double bonds (linoleic, linolenic, and arachidonic) (Fig. 11.9) are among the essential ingredients of food, because they cannot be synthesized in the body. Essential fatty acids play an important role in the body because they serve as the building blocks of many hormones, especially prostaglandins, leukotrienes, thromboxanes, and others, and are also essential components of cell membranes. Essential fatty acids are crucial in metabolism and are crucial for good health. Omega-3 fatty acids contain useful EPA (eicosapentaenoic) and DHA (docosahexaenoic) fatty acids, which are mostly found in marine blue fish. Lack of essential fatty acids causes water loss, dermatosis, and peripheral neuropathy.

Omega-6 is more distributed in plants than omega-3. Animals that are higher on the mammalian evolutionary scale cannot convert omega-6 to omega-3. People can add double bonds and extend alpha linoleic acid to eicosapentaenoic acid and docosahexaenoic acid, but only when the omega-6/omega-3 ratio is low. An excess of omega-6 fatty acids in the diet prevents the conversion of alpha linoleic acid to longer eicosapentaenoic acid and DHA. These types of fats are very easily subject to oxidation reactions, which is why they are packaged in dark, sunproof packaging, to which antioxidants are added during packaging. These substances are important for the work of the heart, kidneys, and liver, the formation of erythrocytes in the bone marrow, etc. Prostaglandins have 6 types, and 1 and 3, among other things, have anti-inflammatory effects. These are special chemical "messengers" used by all tissues in the body.

The nutritional properties of food are determined by their chemical composition, i.e., the content and quality of macro- and micronutrients and biologically active compounds. From the nutritional aspect, it is most important to recognize the category of food, which can be protein-lipid, protein-carbohydrate, protein-carbohydrate-lipid, carbohydrate, and lipid. It can be conditionally assumed that if the content of one of the macronutrients in food is micro- and macronutrient predominant, then that food gets the epithet of the predominant macronutrient.

Edible oils are recognized as essential nutrients in both human and animal diets. From a nutrition point of view, they are concentrated sources of energy providing essential fatty acids which are considered as building blocks for hormones, as well as a carrier medium for the oil-soluble vitamins A, D, E, and K. Different classes of compounds are present in edible oils, such as fatty acids, tocopherols, phenolic compounds, phytosterols, carotenoids, and thioglycosides. The fatty acid composition varies significantly within oils from different plant sources, predominantly depending on the variety but also on the state of ripeness, the area in which the plants are grown, climate conditions, etc. (Ivanova-Petropulos et al. 2015).

11.5 Mineral Composition of Functional Food

Minerals are inorganic compounds and are found in all body tissues and fluids. Depending on the amount in which they are present in the body, these inorganic compounds are divided into minerals, trace elements, and electrolytes. According to this division, calcium and magnesium are minerals, while zinc, copper, and iron are trace elements because they are present in the body in very small amounts. Sodium, potassium, and chlorides are electrolytes by their primary function. The elements, or as we usually call them minerals, are divided into two groups: (1) oligoelements and (2) trace elements.

Oligoelements or macrominerals include those elements whose daily intake is greater than 100 mg. These are Ca, Mg, P, Na, K, S, and Cl.

Trace elements are often divided into several groups:

(a) *Elements necessary for the organism (Fe, Cu, Zn, Mn, I, Se)*. Depending on the required daily intake, these elements can be divided into two subgroups:

    - Elements that we need in the amount of 1–100 mg/day, known as trace elements – "trace elements" (Fe, Zn, Cu, Mn, Si)
    - Elements that we need in an amount of less than 1 mg/day and whose intake is measured in µg/day, known as "ultra-trace elements" (Se, Cr, Mo, B, Sn, V)

(b) *Toxic elements (Pb, Cd, Hg)*

It is important to remember that essential elements can also be toxic. It all depends on the amount that is taken into the body. The most commonly used division of minerals according to the needs of the human body is as follows: (a) electrolytes, (b) macrominerals, and (c) microminerals or trace elements, although no mineral can be excluded from the diet and defined as less necessary.

11.5.1 Mineral Elements

Minerals are inorganic substances. They are naturally found in soil and water, and from there they reach the plant organism. Many minerals are essential substances, so they belong to substances that the human (and animal) organism must take in from food or drink from the outside. Through intake of food and by drinking, they reach the human body in much larger quantities than vitamins. In adults, minerals make up as much as 4% of body weight. Most of them are in the bones. Macrominerals (Greek macros = large) include calcium, magnesium, phosphorus, and sulfur. Microminerals (Greek mikros = small) include those found in very small amounts in our body. These are iron (Fe), zinc (Zn), iodine (I), copper (Cu), manganese (Mn), fluorine (F), chromium (Cr), selenium (Se), molybdenum (Mo), arsenic), nickel (Ni), lithium (Li), vanadium (Va), silicon (Si), and boron (B).

Copper **(Cu, Cu^{+}, Cu^{2+}; lat. Cyprium)** Copper is considered one of the essential trace elements in humans, animals, and many plants. The body of an adult contains 75–150 mg of copper. More than half of the copper in the human body is part of muscles and bones. Its concentration is highest in the liver, brain, kidneys, and heart.

Boron **(B, lat. Borum)** At the beginning of the last century, boron was proven to be an essential mineral for plants, but its significance for humans has only recently been established. Wrinkles are everywhere in the human body, but their highest concentrations are in the bones and tooth enamel.

Zinc **(Zn, lat. Zincum)** Zinc has been known as an essential trace element for plants, animals, and humans since 1930. The average adult has between 1.5 and 3 g of zinc, of which about 60% is found in muscles, 30% in bones, and about 6% in the skin. The highest concentrations of zinc are found in the prostate and sperm of men, as well as in leukocytes and erythrocytes. High concentrations of zinc are also found in the retina of the eye, liver, kidneys, and hair.

Fluoride **(Fluoride, F, F-; lat. Fluo)** Although fluoride is not considered an essential nutrient, it plays a very important role for bones and teeth and is therefore considered beneficial to humans. Fluoride is found in bones, teeth, the thyroid gland and skin. The average adult organism contains about 2.6 g of fluoride.

Phosphorus **(P, PO_4^{3-}, phosphate; lat. Phosphorus)** Phosphorus is the most abundant element in the body after calcium. In the human body, it is approximately 1% of total body weight. It is present in every cell, but it is mostly found in bones and teeth (about 85% of the total phosphorus present).

Iron **(Fe, Fe^{2+}, Fe^{3+}; lat. Ferrum)** Although iron is relatively abundant in the Earth's crust, the human body weighing 70 kg contains only 3–4 g of this mineral. About two-thirds are found in the blood. The therapeutic use of iron dates back several thousand years.

Germanium **(Ge, lat. Germanium)** Germanium, like silicon, is considered by many to be a mineral responsible for the proper mineralization of bones but also an essential antioxidant element. Some authors point out that germanium is actually the key to the action of garlic, ginseng, and medicinal mushrooms (such as shitake mushroom).

Chromium **(Cr, Cr^{3+}, Cr^{6+}; lat. Chromium)** Chromium is an essential microelement. In nature, it is found in three forms which is chromium in the 3+ state – chromium (III). The average adult human body contains from 0.4 to 0.6 mg of chromium, while the elderly have a smaller amount of this microelement. The amount of chromium in the body depends on the environment in which the person lives (and latitude). Many studies show that the number of people with diabetes and heart disease is lower in environments where higher amounts of chromium are ingested.

Iodine **(I, I_2, lat. Iodum)** Iodine is an essential trace element for humans. The average adult contains between 20 and 50 mg of iodine. About 60% of iodine is found in the thyroid gland located at the base of the neck. The rest of the iodine is found in thyroid hormones in the blood, ovaries, and muscles.

Calcium **(Ca, Ca^{2+}; lat. Calcis)** Calcium is the most abundant microelement in the human body. The average adult male contains about 1.5 kg of calcium and a female about 1 kg in the body. About 99% of calcium from the body is found in bones and teeth. The rest is found in the blood, lymph, and other body fluids, cell membranes, as well as in the cell.

Potassium **(K, K^+; lat. Kalium)** Potassium is the main cation (K^+) in the intracellular fluid. So, the main source of K^+ is the cellular material that we receive in food, and that is why potassium deficiency caused by poor nutrition is extremely rare, and it occurs when there are very few protein calories. The average potassium content in the body is about 140 g.

Chlorine **(Cl, lat. Chlorium)** Chlorine is a mineral that is primarily involved in maintaining acid-base balance. It contributes to the buffering capacity in plasma by allowing bicarbonate ions to enter the erythrocytes, without disturbing the electroneutrality of these cells. It is also important for maintaining cell membrane potential and transporting matter at the cellular level. Transmembrane exchange of chlorine with sodium and potassium in the cell maintains a neutral electrical charge and fluid balance needed to maintain a certain pH and osmolarity within the cell. The same role of chlorine is required for normal muscle function, contractions, and conduction of a nerve signal.

Cobalt **(Co, lat. Cobaltum)** Cobalt is very widespread in nature and makes up about 0.001% of the Earth's crust. Although it is a microelement, it causes various disorders in larger quantities. In the 1960s, some breweries used cobalt to regulate sparkling beer. People who consumed such beer to a greater extent experienced nausea, vomiting, and heart problems (cardiomyopathy), which were initially attributed to drunkenness. Cobalt is also often found in polluted air. It is considered an essential microelement and is found in the body within vitamin B_{12}, making it an active part. Apart from its role in this vitamin, cobalt participates in only a few enzymes, which is why some authors do not classify it as an essential microelement.

Magnesium **(Mg, Mg^{2+}; lat. Magnesia)** Magnesium is one of the most abundant minerals in soft tissues. The average adult contains 20–28 g of magnesium, of which about 60% is found in bones. The rest is in the muscles, soft tissue, and body fluids. Magnesium can be found in high concentrations inside cells, especially the heart and brain.

Manganese **(Mn, lat. Mangnes-magnet)** Manganese is an essential trace element for humans. The average adult organism contains 12–20 mg, and it is mostly found in the bones, liver, kidneys, and heart.

Molybdenum **(Mo, lat. Molybdaenum)** Although molybdenum has been known since ancient times, it has often been replaced with lead. It was first defined in 1778 and produced in 1893. Just a few decades later, serious research on its significance for man began. Apart from humans, molybdenum is also important for the entire ecosystem, as it participates as a cofactor of numerous enzymes involved in the biogeochemical cycles of carbon, nitrogen, and sulfur in nature. Molybdenum is found in the body in very small quantities, but its role is important in many biological processes that include the development of the nervous system, the removal of waste substances from the body through the kidneys, and the production of energy in cells.

Sodium **(Na, Na^{+}; lat. Natrium)** Although sodium is a metal in its free form, as such it is not found in nature but is found in compounds or in ionic form. Sodium ions are needed in small amounts in plants and are much more abundant in humans because of their important role.

Nickel **(Ni, lat. Niccolum)** The importance of nickel for the living world was first discovered in the late 60s of the last century, and until then nickel was considered only as a toxic element for the organism. Since then, nickel has been considered a "possible" essential trace element for humans, but little is still known about its role in the body. Most of the research related to the role of nickel in the body has been done on chickens and rats. It is believed that there are about 10 mg of nickel in the human body.

Selenium **(Se, lat. Selenium)** Selenium was identified as a trace element necessary for man only in the 70s of the last century. The average adult contains about 20 mg of selenium, and most of it is found in the liver, kidneys, heart, and spleen.

Silicon **(Si, lat. Carboneum)** Silicon is the mineral most abundant in the Earth's crust, but the need for silicon in the biological system is not significant. Human needs have not been determined but are estimated at 10–20 mg per day. Most likely, the intake of this amount was determined under normal circumstances.

Sulfur **(S, lat. Sulfur)** Sulfur is necessary for human life. It is part of two amino acids as well as in many important biological compounds, such as, e.g., vitamins. The more important sulfur compounds are sulfuric acid, sulfite acid, their salts, sulfur(IV) oxide, and sulfur(VI) oxide. SO sulfur oxide is also known, but it is extremely unstable. Sulfur occurs both in pure flare and in the form of the minerals sulfide and sulfite. Apart from sulfur ores, sulfur was obtained in large quantities by refining coal and refining industrial ash.

Vanadium **(V, lat. Vanadium)** Vanadium is a metal present in the Earth's crust in the amount of 80 ppm (parts per million) in the form of the minerals patronite (VS_4) and vandanite ($Pb_5(VO_4)_3Cl$). It was named after the Scandinavian goddess Vanadis. Vanadium is found in man's environment and in his food. It occurs in traces in the human body, and it is recommended to take it in the amount of 10 micrograms. Vanadium deficiency does not occur in people who eat regularly and properly. According to some authors, vanadium acts as a mineral that affects testosterone production and as such is important for the health of the reproductive system and all the functions that testosterone and androgen hormones carry.

11.6 Influence of Production Process on Biologically Active Compounds

The raw materials from which biologically active components are obtained can be very different, and the most commonly used are plant extracts, plant foods, animal foods, microbial fermentation products, microorganisms, vitamins, and minerals. Procedures that are used to obtain biologically active components of food are identical to the procedures used to obtain phytochemicals and medicinal substances (synthesis, extraction, fractionation, enzymatic hydrolysis, identification). The incorporation of a biologically active component with a proven beneficial effect on one or more functions in the body forms the basis of the production of functional food (Weststrate et al. 2002). A functional food can be created by adding a biologically active ingredient to a traditional food, which thereby expands its action or shapes a completely new product with a specific composition and physiological action (probiotic fruit juice). In the production of functional foods, attention should be paid to possible interactions between the biologically active ingredient and other ingredients of the food (Diplock et al. 1999). The simultaneous presence of several ingredients can significantly modify the action of the active ingredient. Many biologically active substances are unstable during treatment and standing (Siró et al. 2008). They are subject to many reactions, such as oxidation, hydrolysis, ad thermal degradation, which lead to a decrease in their bioactivity. On the other hand, treatment can lead to the formation of new bioactive compounds (Siró et al. 2008). Some of the newly formed compounds, especially from soy, garlic, tea, and dairy products have been shown to be effective in the prevention and treatment of various diseases. In any case, the beneficial effect of bioactive compounds directly depends on the treatment applied in food production (Siró et al. 2008). A better understanding of the mechanisms and kinetics of reactions of bioactive compounds during the applied treatments opens the possibility of modifying the treatment in terms of reducing their adverse effects, as well as emphasizing the positive effects. In addition, the latest technologies in food production (nonthermal treatment) and storage (modified atmosphere) are becoming more diverse and complex. The result of their application is better maintenance of bioactive substances after the applied treatments. New literature data clearly indicate a link between the applied treatments and bioactive components. Thus, the increase in the bioavailability of lycopene, tomato carotene, was among the first positive effects of thermal treatment to be discovered. Different treatments have a beneficial effect on the activity of glucan, the most studied bioactive ingredient in cereals, increasing its bioavailability.

11.6.1 The Role of Minerals in the Human Body

Minerals as well as vitamins play a very important role in the course of metabolic processes. The biological role of individual elements is reflected in their importance in the process of biomineralization (Ca, Mg, Si, P, and F). They act as active centers of metalloproteins (about 30% of enzymes). They are important for the functioning of cell membranes (Na, K, Ca, and Mg) and are extremely important for regulating the balance of fluids in the body, conducting nerve impulses, muscle contraction, etc. It is known that living organisms selectively choose certain elements from nature, while rejecting others. The human body contains approximately 35 elements. Four of them (hydrogen, oxygen, carbon, and nitrogen) make up 99% of the structure of atoms. Thus, e.g., the most widespread element in the Earth's crust is oxygen (67%), followed by silicon (28%) and aluminum (8%). The remaining 1% of the elements in the human body (with the exception of sulfur) are inorganic or mineral constituents of the body. The seven remaining elements: sodium, potassium, calcium, magnesium, phosphorus, sulfur, and chlorine together represent 0.9% of human weight. Seventeen others make up the remaining 0.1%, some, but not all, of which are considered essential in the diet. These elements occur in measurable concentrations but do not perform essential biological functions. Cadmium is one such example. A newborn baby does not have these elements but gradually accumulates cadmium by eating and breathing, to the extent that during his life, the average person living in an industrial area accumulates milligrams of this element. Not only does cadmium have no essential function in our body, but it is even an undesirable element and potentially harmful. Minerals are extremely effective catalysts for all cellular functions. Some minerals work by increasing the electrical potential of cells. Other minerals act as metalloenzymes, which can then act as enzyme reactors in cells, as signal transformers, and as message carriers. In the human diet, minerals play a role in a number of key bodily functions. Disorders in mineral activities and nutritional deficiency of any of the minerals are very common and result in the occurrence of degenerative conditions.

Among the minerals, a group of primary electrolytes stands out, namely, sodium, potassium, magnesium, sulfate, phosphate, calcium, and chloride. Internal relationships between individual minerals play a very important role in the way enough energy is produced by the body's cells. For example, the ratio of sodium to potassium is primary for the electrical conductivity of the cell membrane. Most often, this ratio is used (in various ways) to identify the function of adrenal activity, digestion, and hormone synthesis. The mineral magnesium is an essential activator for more than 300 enzymatic reactions in the body. It must be present for the body to produce ATP (adenosine triphosphate), which is the main intracellular energy storage or biological energy. Magnesium is known to be required for glucose metabolism and is necessary for a normal insulin response. It is also needed to reduce excessive sympathetic stress, for normal bowel function, and for bone health. It has been shown that when phosphorus levels are reduced, calcium is usually precipitated from body fluids and can accumulate in body tissues. The electrolytes chloride and sodium help stabilize blood pH. Both electrolytes are needed for stomach cells to produce hydrochloric acid. Sodium deficiency can lead to low blood pressure, problems with hormone synthesis, and indigestion.

Traces of the minerals chromium and vanadium play important roles in glucose metabolism in the body. Both trace minerals have been shown to increase insulin sensitivity and thus may play a very important role in insulin resistance and type 2 diabetes. Magnesium is required for three critical enzymatic reactions in glucose metabolism: pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose 1,6 bisphosphatase. In addition, magnesium plays a key enzymatic role in various hormones that have a regulatory role in gluconeogenesis, namely, insulin, glucagon, adrenaline, and cortisol. Magnesium deficiency can adversely affect the functionality and production of pancreatic hormones, primarily insulin and glucagon.

Zinc and copper are key to normal hormone synthesis. Zinc is an activator of 200 enzymatic reactions in the body, is required for pituitary gonadotropin, and is required for testosterone synthesis. Copper acts as a stimulant for several neurotransmitters and is closely related to estrogen. There are correlations between estrogen dominance and copper toxicity. The mineral iodine is crucial for the synthesis of thyroid hormones. Iodine deficiency is known to play an important role in some thyroid conditions. Iodine deficiency results in a decrease in the concentration of T_3 and T_4.

11.6.2 Minerals and Metabolism

Most scientists single out 13 minerals essential for nutrition. These are minerals whose constant deficiencies can cause deterioration of the functioning of the organism (disease), which can be prevented and cured by diet, by adequate food, and/or in a state of severe deficiency, by taking supplements (WHO 2003; Marinangeli and Jones 2010). There are still some issues regarding other minerals. The functioning of the mineral elements can be structural, osmotic, catalytic, and signaling.

One specific mechanism is required for the resorption of copper from the gastrointestinal tract because the copper ion (Cu^{2+}) is practically insoluble. One unidentified low-molecular-weight substance from human saliva and gastric juice forms a complex with Cu^{2+} that is soluble in the pH of intestinal fluid. In the cells of the small intestinal mucosa, copper probably binds to a low-molecular-weight metal-binding protein called metallothionein. Copper enters the plasma where it binds to amino acids, especially histidine, and serum albumin in one place that binds it tightly. Copper is removed from the circulation by the liver within 1 hour. The liver processes copper in two ways. The first is that copper is excreted via the bile into the gastrointestinal tract from which it is not resorbed. The fact is that the homeostasis of copper is maintained exclusively by this excretion in the bile, i.e., the more copper is excreted, the more it is in the feces. Normally there is only trace copper in the urine.

Another way of copper metabolism in the liver is its incorporation into ceruloplasmin, a glycoprotein that is synthesized only in the liver. Ceruloplasmin is one of the copper-dependent ferroxidases. It contains 95% of the total copper plasma. Ceruloplasmin is not a transport protein for Cu^{2+} because the copper from ceruloplasmin does not exchange with the copper ion or copper bound to other molecules. Ceruloplasmin contains 6–8 copper atoms, half in cupro (Cu^{+}) and half in cupra (Cu^{2+}) form.

Ceruloplasmin oxidizes Fe^{2+} to Fe^{3+} which is necessary for the mechanism of iron resorption from the gastrointestinal tract. Other copper metalloproteins include cytochrome oxidase, tyrosinase, monoamine oxidase, superoxide dismutase, and lysyl oxidase. Boron is very efficiently resorbed and excreted in the urine.

Zinc metabolism is also a very complex process in the human body. Zinc-binding factor (picolinic acid), which is secreted by the pancreas, is present in the lumen of the small intestine. In order for picolinic acid to be synthesized, the presence of vitamin B_6 is necessary. The zinc picolinate complex is transported through the cell to the small intestine (intestinum) and then to the liver where it is stored. Vitamin B6 deficiency can, especially in the elderly, lead to reduced zinc resorption. Zinc as well as iron can be bound to a single protein in mucosal cells. It is then transmitted through the serous membrane of the mucosal cell in the serum and binds to albumin. Copper may affect zinc resorption due to competition for serum albumin binding sites. When foods are high in phosphate and calcium, zinc deficiency can be more severe. Zinc is processed in the juice of the pancreas and to a lesser extent in the bile. Therefore, the major route of zinc excretion is via the feces. Significant amounts of zinc can also be lost through sweating, especially in the tropics. When the proportion of zinc (as well as copper) increases, it binds to metallothionein in the liver. Zinc is not stored well enough in the body, so reducing zinc intake through food can lead to zinc deficiency in the body relatively quickly. The level of zinc in the body can best be determined by measuring it in erythrocytes and leukocytes. Studies from 1997 showed that zinc resorption doubles during lactation, which enables better milk synthesis, but there is no data that this happens during pregnancy. Hormone therapy can often be the cause of poor zinc resorption. In estrogen treatment in postmenopausal women, zinc resorption decreases by 35% after only 3 months of treatment.

Phosphorus enters the body through food and is resorbed in the small intestine. Any excess phosphorus is excreted from the body through the kidneys. Regulation of calcium and phosphorus in the blood is performed by parathyroid hormone (PTH) and vitamin D. A slight decrease in blood calcium (e.g., in case of insufficient intake of calcium in the body) leads to parathyroid glands increasing the secretion of PTH. PTH stimulates the translation of vitamin D in the kidney into its active form. Increased levels of calcium in the blood lead to increased intestinal resorption of calcium and phosphorus. Stimulation of PTH leads to decreased urinary calcium excretion and increased phosphorus excretion.

Iron is mainly found in food in ferrous form (Fe^{3+}) and is tightly bound to organic molecules. In the stomach, where the pH is lower than 4, Fe^{3+} can dissociate and react with low-molecular-weight compounds such as fructose, ascorbic acid, citric acid, and amino acids to form complexes that allow Fe^{3+} to remain soluble at neutral pH such as in the small intestine. Iron does not leave the heme in the stomach but as such passes into the small intestine.

In healthy people, only 5–10% of iron is absorbed from food. In childhood, the absorption is greatest and decreases with age. In foods of animal origin, iron is present in the form of organic heme iron, while in plant foods it is found in the form of inorganic nonheme iron. These two types of iron are resorbed into different pathways. 20–30% of heme iron can be absorbed from food, as opposed to 2–5% of nonheme iron. If vitamin C is also taken in through food, then the percentage of adopted nonheme iron increases to about 50%. Vitamin A and beta-carotene can also increase the uptake of nonheme iron. Iron must be in ferro form in order to be resorbed, and the hydrochloric acid found in the stomach translates the ferric form of iron into ferro. Iron resorption is a slow process that takes 2–4 hours. If the level of iron in the body is low, then its resorption increases. In this case, the level of resorption can increase by 10–20%. Different factors affect iron resorption, and the amount of iron that will be absorbed from food depends on the mutual interactions of these factors. Sugars and amino acids can increase resorption, while zinc, oxalates, and green vegetables such as spinach, tannins from tea, and coffee can reduce iron resorption. Phytates and unpolished cereals can reduce resorption, although the opposite effect can occur in the presence of meat and vitamin C. Milk proteins, albumin, and soy proteins can also reduce resorption. The normal daily loss of iron from the body is only up to 1 mg. Women also lose iron in their menstrual blood. Therefore, the only way to regulate the total amount of iron in the body is iron resorption. When the condition of the organism is normal, 10–20 mg of this mineral is obtained through food every day. However, less than 10% is resorbed in the body. Thus, under normal conditions, very little iron is resorbed from food, and the amounts excreted in the urine are minimal. At the same time, much of the total iron in the body is continuously redistributed to various parts of the body through several metabolic circuits. The greatest needs for iron are in childhood and puberty. Children at these stages of development resorb a higher percentage of iron from food than adults. Iron deficiency in childhood and puberty, as well as in women with menstruation, can be attributed to iron deficiency in food. If the deficiency occurs in adults, it can usually be attributed to significant bleeding (WHO 2003).

11.7 Instrumental Techniques for Functional Food Characterization

According to their chemical composition, food is a mixture of different compounds, which are of organic and/or inorganic origin. These compounds in food can be of natural or artificial origin. Determining the content of these compounds in food is crucial for assessing the quality and safety of food.

For food analysis, the following are used: physical, physicochemical, chemical, microbiological, and enzymatic methods of determination. This chapter discusses for the most commonly used instrumental methods, which belong to the physicochemical methods of determining the quality and safety of food. The development of analytical strategy and methodology, which is closely related to control during primary food production and during its processing and marketing, as well as with relevant legislation, is an imperative which on the one hand provides consumers with quality and safe food and, on the other hand, through the harmonization of domestic legislation with international regulations in this area, the inclusion of our country in international trade flows is ensured. Given the wide range of methods and techniques used today in the control of food safety, it is very important that the analytical result is reliable and unambiguous, i.e., confirmed by the confirmatory method. It is also important that analytical methods, whether those used for sample triage or confirmatory, whether multiresidual or for individual determinations, be selective, sensitive, accurate, and precise.

Instrumental methods of qualitative and quantitative analysis of chemical substances today are very numerous and diverse and occupy a significant place in analytical research. Instrumental analyses measure some of the physical properties of a substance and, based on that, determine its chemical composition. Lately, they are increasingly used because almost every physical property of a compound can be the starting point for the development of an instrumental technique. The main reasons why instrumental analyses are very important today are time-savings, often greater accuracy in operation, the ability to analyze very small samples, and the ability to avoid often very complicated chemical separations.

Many of the food analysis methods used today are based on the basics of analytics that were discovered a hundred or more years ago. Most of the applied analyses used to determine the basic parameters of food quality are based on the choice of a rapid analytical procedure for individual parameters without the need to use sophisticated equipment and chemicals. Furthermore, these days, quality control and characterization of food product usually are conducted with fast, efficient, multivariable determination instruments.

11.7.1 Applied Spectroscopy

The spectroscopic techniques used are fast, accurate, and, most importantly, cheap, and they can be used to control milk, dairy products, meat, meat products, and other foods. Rapid near-infrared spectroscopy in routine analytics and process control replaces common, expensive, and demanding classical analytical methods. These methods are most commonly used for qualitative and quantitative analysis of milk and dairy product ingredients as well as for determining the total number of bacteria in raw milk. It is of paramount importance in the analysis and research of substances. Spectroscopic techniques form the most important group of techniques in instrumental chemical analysis. According to the principle on which they are based and performed, spectroscopic techniques can be applied in the laboratory, in an industrial plant (process analysis), or in the field. The limit of identification of individual spectroscopic techniques is different and ranges in a very wide range, from a few picograms to a few grams. The use and application of spectroscopic techniques in the food industry are growing, and the answers they provide make it easier to solve problems in food production and distribution.

Ultraviolet/visible (UV/Vis) spectrophotometry is a spectroscopic method that involves studying the absorption of electromagnetic radiation in the range between 200 and 800 nm. As a large number of organic compounds are not absorbed in this part of the spectrum, UV/Vis spectrophotometry, compared to other structural methods (IC, NMR, MS), has far less application for structural determinations and is mainly used as a complementary method for identifying parts of molecules which are absorb in the said area, the so-called chromophores. The UV/VIS spectra obtained in this way provide very useful information on the structure of the test compound. For example, it is an indispensable auxiliary (and often the main) method for identifying natural conjugate compounds (such as plant pigments (carotenoids), polyacetylenes, porphyrins, total phenols, flavonoids, total anthocyanins, monomeric anthocyanins), percentage of polymer color, total antioxidant activity, etc. In addition to its application for the identification of organic compounds, UV/Vis spectrophotometry is widely used today in quantitative analysis. Its advantages over other methods are its extremely high sensitivity and easy operation of the instrument.

Infrared radiation (IR) is electromagnetic radiation of wavelengths from 0.7 to 500 mm. Its name derives from the fact that the energies of infrared radiation are less than the energies of the visible part of the spectrum to which they continue. The IR method has many advantages because they allow fast measurement of a large number of quantities without destroying the sample. Near-infrared radiation affects the stretching vibrations of covalent bonds C–H, N–H, and O–H in the molecule. Near-infrared spectroscopy techniques use the near-infrared reflection (NIR) and near-infrared emission (NIT) techniques. These techniques can be used to analyze liquids and solids such as milk, cheeses, and milk powder.

Atomic absorption spectrophotometry (AAS) is an absorption method that measures the decrease in the intensity of monochromatic radiation as it passes through the atomic vapor of a sample. Hollow cathode lamps made of the element to be determined are used as the radiation source. The samples are evaporated at temperatures from 2000 to 6000 K, and then the absorption of electromagnetic radiation of the appropriate wavelength is measured. Due to the high sensitivity, it is possible to determine more elements from the solution even in a very low concentration range. AAS is primarily used to determine the concentration of metals in foods such as metals in plant material and plant extracts. Samples that are already liquid (e.g., fruit juices, wine) can be easily analyzed without prior preparation, i.e., after dilution with water or a reagent. The basic prerequisite for analysis by atomic absorption spectrophotometry is that the sample be homogeneous and at least in a semiliquid state. Most often, concentrations are determined from a calibration diagram obtained using a series of standard solutions of the analyzed element of known concentrations. Newer AASs are equipped with a computer device that easily programs the analysis parameters and prints the measurement results. The instrument measures each standard, i.e., sample, three times, conducts measurement statistics, constructs a calibration diagram, and calculates the concentration of an unknown sample. Important advantages of AAS are primarily the analysis of about 65 different elements in a whole range of different samples; low limits of detection (mg/cm^3, mg/dm^3, ng/dm^3) and possible analysis of trace metals and small amounts of samples; and high precision and accuracy; if the samples are a solution or fine suspension, the pretreatment of the samples is practically superfluous, and their direct analysis is possible; AAS is more sensitive to determinations, e.g., Ca, Mg, Cu, Fe, Zn, Pb, Hg, and Cd in biological material. High sensitivity allows analysis of analyte traces, analysis of small amounts of material, and dilution of solutions which reduces interference.

Atomic emission spectrophotometry (AES) is most commonly used in multielement analysis and quantification. Atoms, especially metals, emit electromagnetic radiation when they are energized to excite their external electrons. Since electrons can only exist at certain energy levels, specific to each element, the wavelength of the emitted radiation itself is characteristic of a given element. This characteristic emission gives the opportunity not only for qualitative but also for quantitative analysis, because the intensity of the emitted radiation is directly proportional to the number of atoms or ions that is emitted. The emission of light from each element, with a certain wavelength, allows this physical phenomenon to be used as a very sensitive and specific method of analysis. It is characteristic that the external electrons of the atoms of many metals are more easily excited than those of nonmetals (Linge 2009).

The basic principles on which emission spectroscopy is based are the acquisition of line spectra emitted by excited atoms or ions in the vapor state after the absorption of adequate energy. Electronic levels are quantized and under normal conditions are in the lowest energy state, known as the normal or ground state. Sufficient energy is delivered to the atom or ion, thermally or by means of an electric source; one or more electrons can be converted to a higher energy state. The excited atom is held for a very short time, and when the electrons return from the higher energy state to the ground state, radiation is emitted.

When the atom returns to its ground state, the electron can make several specific jumps or energy changes, emitting radiation of different wavelengths. When using high-energy excitation sources, a larger number of lines usually appear, the intensity of which depends on the number of atoms emitting energy and the amount of energy. The number of lines is determined by the number of transitions from different levels and the intensity by the number of electrons emitting energy.

In emission methods, arc, spark, flame, or plasma can be used as excitation or excitation agents for atoms. Emission tests obtained from arc, spark, or argon plasma indicate the existence of three types of spectra: continuous, band, and linear. At arc and spark sources, continuous radiation is emitted from the heated particles obtained from the surface of the electrodes. Band spectra, obtained from a series of close lines, are observed in some wavelength ranges, especially in arc and spark sources. Emission spectrometry is based on linear spectra obtained by excitation of atoms and ions. Atomic line spectra are important from the point of view of analytical purposes, because there are no identical line spectra for two elements.

If molecules or atoms in the gaseous state are provided with sufficient energy, the chemical bonds are broken. The result of this process is the creation of an ionized gas called plasma. Plasma is composed of a large number of free electrons and a number of free positively charged ions, i.e., plasma is electrically neutral and is the fourth aggregate state of matter. Plasma means partially and completely ionized gases. The ions created in the plasma are able to absorb enough energy from an external source to maintain the temperature at a level at which further ionization keeps the plasma stable and a temperature higher than 10,000 K. This state of matter contains a large amount of energy per unit volume and is therefore a suitable excitation source in emission spectrometry. Microscopically, plasma behaves like any other gas, and macroscopically it behaves like a liquid: it conducts electricity and transmits different types of waves. Laboratory plasma is produced from a gas (usually argon) under reduced atmospheric pressure. Given the characteristics of plasma, ions and electrons would be difficult to maintain as a whole, which is actually achieved with an electromagnetic field. The main components of such an instrument are plasma-generating system, spectrometer, and computer system. The plasma-generating system consists of a plasma chamber, a radiofrequency vent generator, and an input system of the analyte (disperser and spray chamber). The plasma chamber consists of three concentric quartz tubes. Around the top of this tube is an induced coil connected to a radiofrequency generator. The ionization of argon is initiated by a spark from a narrow coil. The resulting ions and the electrons attached to them interact with the fluctuating magnetic field obtained from the inductive coil. This interaction causes the ions and electrons to be directed in a closed circular path. The temperature of the plasma thus created is high enough to require thermal insulation of the outer quartz tube, which is made possible by the introduction of argon tangentially around the tube walls.

Mass spectrometry with inductively coupled plasma (MS-ICP) is the leader technique in the multielement determinations in various samples. The basic components of an inductively coupled plasma mass spectrometer are (Ammann 2007):

- Sample introduction system
- Generation of ions using inductively coupled plasma
- Plasma/introductory part in vacuum
- Ion-focusing system
- Ion separation and measurement system

Sample introduction system The sample is introduced into the inductively coupled plasma (ICP) in the form of an aerosol, which is formed as a result of passing the liquid sample through a simple pneumatic nebulizer. Larger aerosol particles are removed from the spray chamber through a special waste drain. Only smaller aerosol particles are carried by the flow of plasma argon (Fig. 11.3). The temperature in the spray chamber is precisely controlled by a thermoelectric device to prevent signal changes that could be caused by large changes in temperature as well as to reduce the passage of large amounts of fluid into the plasma (Linge and Jarvis 2009).

Production of ions in inductively coupled plasma (ICP) The generated aerosol passes into the plasma, which is made possible by a special supply of argon (argon carrier beam) through a glass attached part marked as a torch. The torch is located in the center of a copper coil through which an electric current with high power and frequency passes. The generated magnetic field causes collisions between free electrons and argon atoms, resulting in the formation of ions and more electrons. The generated energy of the radiofrequency (RF) generator is up to 1600 W. Two operating frequencies are recommended according to Linge (2009) for maximum power of inductively coupled plasma, namely, 40.68 MHz and 27.12 MHz, which result in higher plasma temperatures. The high plasma temperature (up to 10,000 K maximum and about 7500 K in the central part) allows rapid drying of the aerosol droplets, atomization, and ionization by removing one electron from each atom. The ions formed, which are formed within about 10 ms of the aerosol entering the plasma, are positioned at about 7 mm in the plasma (Ammann 2007).

Plasma/introductory part in vacuum The positively charged ions generated in the plasma are extracted into a vacuum system using metal cones. These cones are special metal plates with central openings (<1 mm) allowing high vacuum to be maintained between the input system and the mass spectrometer.

Ion focusing system The ions created in the plasma that pass through the holes of the cones are focused with the help of electrostatic lenses and thus pass into the system which is under high vacuum (where the mass spectrometer is located). Extraction lenses are a secondary essential part that allows ions to be separated from photons and residual neutral atoms (Ammann 2007; Linge and Jarvis 2009).

Ion separation and measurement system In inductively coupled plasma mass spectrometry, three types of mass spectrometers are used: quadrupole, magnetic sector, and TOF (time-of-flight). The most commonly used mass spectrometer is the quadrupole, which uses a combination of DC (direct current) and AC (alternating current) electrostatic fields to separate ions based on their mass/charge ratio (m/z). Since almost only single ions are generated in plasma, the mass/charge ratio is identical to the mass of the ion, creating a very simple mass spectrum for interpretation. The DC/AC electrostatic field ratio is fixed but the spectrometer voltages can be varied. For a given voltage tuning, only one isotope is stable, and the quadrupole quickly scans in the 2–260 amu mass range, missing any mass of interest to the electronic multiplier (EM). An electronic multiplier is a device that detects any ions that are leaked through the quadrupole. The detector calculates each signal for one isotope, thus creating a mass spectrum. The mass spectrum is a simple and accurate qualitative representation of the analyzed sample. The size of each peak is directly proportional to the concentration of one element in the sample. The quantitative result is created by comparing the signal of interest with the signals obtained for the calibration standards for the respective elements (Ammann 2007).

Comparative analysis for AES-ICP and MS-ICP The use of these two spectrometric methods has shown that both techniques are characterized by certain strengths and weaknesses. The sensitivity of AES-ICP systems is much lower than MS-ICP when it comes to total dissolved solid (TDS). Particular care should be taken when using mass spectrometry, where TDS is required to range within 0.1–0.4%. Therefore, in atomic emission spectrometry, the total dissolved salts in the sample can range up to 20%. This suggests that certain samples require a large dilution or an initial small sample, which can sometimes affect the accuracy of the results. Both techniques are multielement, with a dynamic determination area for AES-ICP of 106 and for MS-ICP of 108 (Linge 2009). Both techniques are suitable for semiquantitative and isotopic analysis. Mass spectrometry shows a larger distribution of the multielement determination range (82 elements) as opposed to atomic emission spectrometry with inductively coupled plasma (73 elements). Mass spec-

Table 11.1 Comparative analysis for the sensitivity of ICP-AES and ICP-MS

Element	ICP-AES with radial plasma, LOD in ppb	ICP-AES with axial plasma, LOD in ppb	ICP-MS with quadrupole in LOD ppt
Ag	2	0.5	0.01–0.1
Al	6	1.5	0.1–10
As	12	2	1–10
Au	6	0.6	0.01–0.1
B	0.5	0.2	10–100
Ba	0.2	0.04	0.01–0.1
Be	0.2	0.06	0.1–1
Bi	18	2	0.01–0.1
Ca	0.03	0.03	1–100
Cd	1	0.1	0.01–0.1
Co	2	0.5	0.1–1
Cr	2	0.4	0.1–1
Cs	3200	/	0.01–0.1
Cu	2	0.3	0.1–1
Dy	0.3	/	0.01–0.1
Er	0.7	/	0.01–0.1
Eu	0.3	/	0.01–0.1
Fe	1	0.3	0.1–100
Ga	7	/	0.1–10
Ge	10	/	1–10
In	10	/	0.001–0.1
K	6.5	0.5	0.1–100
La	0.02	/	0.01–0.1
Li	1	/	0.01–0.1
Mg	0.1	0.03	0.1–1
Mn	0.3	0.05	0.1–01
Mo	4	0.5	0.01–0.1
Na	1	0.2	0.1–100

trometry takes precedence over atomic emission over the limit of detection and quantification for all elements to which it is applicable. Table 11.1 provides an overview of the detection limits compared to both techniques. The comparative analysis is given for both types of atomic emission technique (with radial and axial plasma) and mass spectrometry with quadrupole as mass spectrometer. This review is not specific to all types of techniques mentioned above. These values vary depending on the performance and specifications of each model of technique used.

The sensitivity of the above techniques largely depends on spectral and nonspectral interferences. In inductively coupled plasma atomic emission spectrometry, spectral interference is common due to the richness of the spectral lines produced by the hot plasma. If the spectral interferences for a given element are high, for a given wavelength, an optional method is to select an alternative spectral line, although this may reduce the sensitivity of the method. Spectral interferences in mass spectrometry are much rarer and mostly often occur as isobaric interferences (oxides, double-charged ions, etc.). On the other hand, the effect of the analysis matrix is much more significant in MS-ICP than in AES-ICP, which is minimized.

11.7.2 Applied Chromatography

Chromatography is a general term applied to a wide variety of separation techniques based on the partitioning or distribution of a sample (solute) between a moving or mobile phase and a fixed or stationary phase. Chromatography may be viewed as a series of equilibrations between the mobile and stationary phase. The relative interaction of a solute with these two phases is described by the partition (K) or distribution (D) coefficient (ratio of concentration of solute in stationary phase to concentration of solute in mobile phase). Stationary phase in chromatography is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid, it is termed as liquid chromatography (LC), and if it is gas, then it is called gas chromatography (GC). Gas chromatography is applied for gases and mixtures of volatile liquids and solid material. Liquid chromatography is used especially for thermal unstable and nonvolatile samples (Snyder et al. 2009).

Liquid chromatography (LC) is a physical separation technique for trace analysis. It is based on the interaction of an analyte with a stationary phase (column with particles) and a mobile phase (liquid eluent or a mixture of eluents). There are several liquid chromatography techniques applied in food analysis, namely, paper chromatography, thin layer chromatography (TLC) (both of these techniques may be referred to as planar chromatography), and column liquid chromatography, all of which involve a liquid mobile phase and either a solid or a liquid stationary phase. However, the physical form of the stationary phase is quite different in each case.

High-performance liquid chromatography (HPLC) is one of several chromatographic methods for the separation and analysis of chemical mixtures. This technique is exceptional in terms of the following characteristics:

- Almost universal applicability, few samples are excluded from the possibility of HPLC separation.
- Remarkable assay precision (±0.5% or better in many cases).
- A wide range of equipment, columns, and other materials is commercially available, allowing the use of HPLC for almost every application.
- Most laboratories that deal with a need for analyzing chemical mixtures are equipped for HPLC.
- It is often the first choice of technique (Snyder et al. 2009).

High-performance liquid chromatography (HPLC) has been significantly improved in terms of selectivity and resolution of mixture components. This was achieved by applying a column filled with a stationary phase composed of spherical microparticles with a diameter of 2–5 μm or porous monolithic materials that significantly lead to a drop in pressure in the column. HPLC uses the selected structural property of the substances and does not change their chemical nature during the analysis. Depending on the chemical structure, the ingredients of the mixture spend different time in the column because they have different retention affinities on the stationary phase.

Using this chromatography technique, it is possible to perform structural and functional analysis and purification of many molecules within a short time. This technique yields perfect results in the separation and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules. In HPLC, mobile phase passes through columns under 10–400 atmospheric pressure and with a high (0.1–5 cm/sec) flow rate. In this technique, the use of small particles and application of high pressure on the rate of solvent flow increases separation power of HPLC, and the analysis is completed within a short time.

In liquid chromatography, the choice of detection approach is critical in order to guarantee that all the components are detected. The most widely applied detectors are UV detector, which is capable of monitoring several wavelengths concurrently, photodiode array (PDA) detector, refractive index detector (RID), and mass spectrometry detector (MS). In the case of PDA, a wavelength range can be programed and all the compounds that absorb within this range can be identified in a single analysis. The PDA detector can also analyze peak purity by matching spectra within a peak and finds its application in the method development. The refractive index detector is the detector of choice when one needs to detect analytes with restricted or no UV absorption, such as alcohols, sugars, carbohydrates, fatty acids, and polymers. Decent trace detection performance is secured through a low noise. This detector has the lowest sensitivity among all detectors but is suitable at high-analyte concentrations. Mass spectrometers operate by converting the analyte molecules to a charged (ionized) state, with subsequent analysis of the ions and any fragment ions that are produced during the ionization process, on the basis of their mass to charge ratio (*m/z*). Several different technologies are available for both ionization and ion analysis, resulting in many different types of mass spectrometers with different combinations of these two processes. The two most widely used ionization interface systems are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Which one will be chosen depends on the physicochemical properties of the analytes (i.e., polarity and acidity). Ionization takes place at atmospheric pressure, and both are considered to be a soft ionization method, i.e., the mass spectrum provides mainly the molecular weight information, unless fragmentation techniques are used. Since compounds partly co-elute from the chromatographic system, the clear assignment of the individual fragments cannot be accomplished using only LC-MS, where only molecular ion masses are available. To overcome this problem, tandem mass spectrometry (MS/MS), which involves multiple steps of mass selection or analysis, is nowadays mainly used (LC/MS/MS or LC/MSn). These systems are able to determine residues in the lower ppt range.

Among the different methods, reversed-phase high-performance liquid chromatography (RP-HPLC) is commonly employed for the separation of complex mixtures of phenolic compounds present in wine and grape using C18 column, UV/Vis diode-array detector, and a binary solvent system with an acidified polar solvent such as aqueous solution of acetic, perchloric, phosphoric, or formic acids (solvent A) and a possibly acidified organic modifier such as methanol or acetonitrile (solvent B). Phenolic compounds show characteristic absorbances in the UV/Vis region: anthocyanins have an absorbance maximum around 520 nm, flavonols around 360 nm, and hydroxycinnamic acids at 320 nm. Flavan-3-ols can be detected at 280 nm, and these substances have fluorescence properties that the other wine polyphenols do not. Liquid chromatography coupled to mass spectrometry, as a sophisticated technique, has been used for the characterization of phenolic compounds in wine samples that allows a variety of phenolic structures to be identified (Ivanova et al. 2011a).

Gas chromatography (GC) is used widely in applications involving food analysis. Typical applications pertain to the quantitative and/or qualitative analysis of food composition, natural products, food additives, flavor and aroma components, a variety of transformation products, and contaminants, such as pesticides, fumigants, environmental pollutants, natural toxins, veterinary drugs, and packaging materials (Lehotay 2002). Typically, GC is useful for analyzing nonpolar and semipolar, volatile and semi-volatile chemicals. Without chemical derivatization, GC is often used for the analysis of sterols, oils, low-chain fatty acids, aroma components and off-flavors, and many contaminants, such as pesticides, industrial pollutants, and certain types of drugs in foods.

Separation of the components from the mixture by gas chromatography (GC) is based on the difference in the partition coefficients between the stationary liquid and mobile gas phases. The basic principal of gas chromatography is that the greater the affinity of the compound for the stationary phase, the more the compound will be retained by the column and the longer it will be before it is eluted and detected. Thus the heart of the gas chromatograph is the column in which separation of the component takes place, and to this must be added the source and control of the carrier gas flow through the column, a means of sample introduction and a means of detection of the components as they elute from the end of the column. Since temperature will influence the volatility of the analytes, the column is placed in a thermostatically controlled oven.

Gas chromatography (GC) is a technique most commonly used in combination with mass spectrometry (MS), and if it is used for product identification (under very controlled conditions), it must be directly coupled to a mass spectrometer when information other than a comparative fingerprint (program) is required, such as positive identification of peaks on the chromatogram. Complex mixtures can be very easily separated by gas chromatography, and MS is used to identify individual components, because the mass spectrum provides information about their structure. The individual components of the mixture appear on the gas chromatogram in the form of separate peaks. Retention time can serve as a quantity for qualitative definition, but this is not a reliable way, so it should in no way be used to determine the composition of unknown and previously unidentified compounds.

Estimation of the aroma compounds in wine is usually performed by gas chromatography/mass spectrometry (GC/MS), as a highly efficient separation technique for volatiles' analysis. Moreover, GC/MS is also suitable for quantification purposes, using polar column for separation of the components, since it is more sensitive for analysis of components present in a low concentration, as well as in a complex matrices, as wine. The volatile compounds are usually extracted by different methods, such as solid-phase extraction, solid-phase microextraction, stir bar sorptive extraction, or liquid-liquid extraction methods using organic solvents before the gas chromatographic analysis (Ivanova et al. 2012, 2013; Ivanova Petropulos et al. 2014a).

Gas chromatography (GC) is the technique of choice for the analysis of fatty acids in edible oils, usually coupled with a flame ionization detector (FID) or for the analysis of volatile compounds (Ivanova-Petropulos et al. 2015; Murkovic et al. 1996). GC or HPLC in combination with mass spectrometry, as sophisticated techniques allowing structural identification and quantification by single-ion monitoring (SIM) or multiple-ion monitoring (MIM) of different classes of compounds, is used for the analysis of different classes of compounds present in the oils. Recently, a headspace comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (headspace GC × GC-TOF/MS) was used for the classification of volatiles from vegetable oils in order to build a statistical model that should help to identify adulteration of oils (Hu et al. 2014).

11.8 Application of MALDI-TOF-MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a powerful new technique that has a great potential in food analysis, as well as on wine and grape analysis (Sugui et al. 1998; Wang et al. 1999; Reed et al. 2005; Ivanova et al. 2011b; Ivanova Petropulos et al. 2014a, b). This technique allows successful determination of the molecular weights in complex samples directly from ion abundances in the mass spectrum without previous isolation or cleanup of the sample (Ivanova et al. 2011b). MALDI-MS has also been applied to carbohydrates (Mock et al. 1991) and fructooligosaccharides in plants and food samples (Wang et al. 1999), and it has proved to be highly suited for the analysis of highly polydisperse and heterogeneous proanthocyanidins (Monagas et al. 2010). In addition, MALDI-TOF-MS has been used for the identification of wine and grape anthocyanins and confirmation of the dominant anthocyanin compounds such as malvidin and its derivatives, using different matrices (2,5-dihydroxybenzoic acid (2,5-DHB), α-cyano-4-hydroxycinnamic acid (CHCA), and sinapic acid (SA)), as well as C70 fullerene applied for the first time for this purpose, without sample preparation (Ivanova et al. 2011b).

Coupling of MALDI with one of the simplest mass analyzers, time-of-flight (TOF), allowed sensitive and efficient technique with high sensitivity, ease of use, speed of analysis, good tolerance toward contaminants, and, the most important, the ability to analyze complex mixtures, giving access to a theoretically unlimited mass range. A basic characteristic of MALDI is mixing the analyte with a suitable matrix, followed by application of the mixture on a MALDI plate and then irradiation by a pulsed laser. During the irradiation, the analyte and matrix molecules are ablated and ionized. A number of substances have been tested and applied as MALDI matrices, but there are no general rules for predicting the suitability of a substance as matrix. The most frequently used matrices are derivatives of benzoic acid, e.g., 2,5-dihydroxybenzoic acid (2,5-DHB), and derivatives of cinnamic acid, e.g., α-cyano-4-hydroxycinnamic acid (CHCA) or sinapic acid (SA). Fullerenes have also been used as matrices, whereas the first application was for laser desorption of biomolecules involving application of protein analyte solution directly onto the predeposited fullerene film. The most widely used sample preparation method for MALDI analysis is the dried droplet technique when a mixed solution of sample or analyte/s and matrix is deposited onto a MALDI plate and allowed to dry and the sandwich method where the sample is placed “in a sandwich” between two matrix layers (Ivanova et al. 2011b; Ivanova Petropulos et al. 2014a, b).

11.9 Application of Immunochemical Methods in Food Analysis

As one of the tests for the detection of pathogens in food based on immunological characteristics, the enzyme-linked immunosorbent assay (ELISA) is widely used (Crowther 1995). The method is designed to replace detection or isolation on a solid substrate, is relatively easy to perform, can be applied to a large number of pathogens, can be semiautomatic, and gives a quick result. Depending on the needs, the goal of this test may be to detect the genus, species, or serotype of the microorganism. However, a positive result obtained by an ELISA test must be confirmed by a conventional test. The biggest advantage of this method is the performance of negative screening, i.e., the possibility of including a significantly larger number of samples that can be tested for the presence or absence of a certain pathogen, assuming that there is an acceptable level of false negative results (Crowther 1995). Positive results obtained by ELISA techniques must be confirmed by analytical techniques such as aqueous chromatography or gas chromatography and with fluorescent, ultraviolet, or concentrated spectrophotometric detection techniques (Crowther 1995).

The usefulness of the ELISA method has been confirmed by many authors in proving the types of meat and various plant proteins often used as additional ingredients in meat production, for the detection of enrofloxacin residues in chicken muscle and liver. Using appropriate antibodies and standards, ELISA can be used to quantify soy proteins, pea proteins, and gluten in heat-treated products. The ELISA method has been tested several times for the purpose of qualitative and quantitative determination of denatured soy proteins (Crowther 1995).

11.10 Conclusion and Perspectives

Rapid technological development and negative environmental problems affect every segment of the biosphere. A special aspect is the availability of safe and quality food for the human population. Functional food is an efficient product of nature, but its creation is certainly subject to existing environmental factors of pollution. That is why in this chapter we have given an overview of the functional and nutritional characteristics of the important biologically active components of the functional food, but also a special section is dedicated to the analytical techniques for its chemical characterization. Chemical characterization is essential in determining the composition of nutrients, but on the other hand, it is a necessary tool in determining its innocence. A great benefit of today is the availability of modern, fast, accurate, precise, and sensitive analytical techniques, which allow determining not only the functional and nutritional components of food but also the presence of toxic and unsafe chemicals, in order to control the quality and food safety.

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Chapter 12
Characterization of Multi-element Profiles and Multi-isotope Ratio Records as a Tool for Determination of the Geographical Origin of Various Plant Species

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Abstract Determination of food authenticity is an important issue in quality control and food safety. In recent years, many serious diseases appeared related to foodstuffs, so providing the motivation for the scientific community to work more intensively in this area. Authenticity is a quality criterion for food and food ingredients and is required more and more worldwide, as a result of legislative protection for regional foods. Reviews of analytical methods for the determination of geographical origin of food and beverages have been published. However, organic components of a food crop production depend on various conditions (e.g. fertilization, history of the field, climatic conditions in the year of cultivation, geographic location and soil composition), so it is not always possible to determine the origin of a product by analysing the organic components. Additionally, methods based on elemental composition have been reviewed as have methods based on isotope ratios. Over the past decade, with the development of new advanced analytical techniques [e.g. thermal ionization MS (TIMS), inductively coupled plasma MS (ICP-MS) and dynamic reaction cell-ICPMS (DRC-ICPMS)], we can successfully retrieve elemental and isotopic compositions of any given food sample and determine the geographic origin successfully. The growing concern of the consumers stimulated

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scientific research and publications in recent years, including multi-element and isotope ratio methods of analysis in food authentication after statistical evaluation of the results. Several aspects will be described: (a) propose a new technique for evaluating spatially explicit trace element profile in various environmental samples (with organic and inorganic complex matrixes); (b) evaluate whether element composition of the environmental samples can be attributed to large-scale geographic trace element variation associated with underlying geology or fine-scale spatial differences related to foraging habitat; (c) determine whether element profiles along the individual species are autocorrelated (i.e. occurring in a predictable or random pattern); (d) prove spatially resolved multi-element information that could be used for identification of geographical origin of various environmental samples and foodstuffs.

Keywords Isotopic ratio · Trace elements · Foodstuff · Mass spectrometry

12.1 Introduction

Historically, food products have always been linked with a specific geographical origin. Food consumption habits were created by the local natural resources and the social or cultural factors of the community. Such links have disappeared over the time for various reasons, mostly because of the globalization of the food industry, following the extensive growth in technological means over the past century. Thus, food consumption in a region no longer necessarily reflects food production of that area. However, in recent years, consumers have renewed their interest in food strongly identified with a place of origin (Giovannucci et al. 2009). The reasons for this increasing interest of consumers vary from the global trend for organic and health products to their concern about animal welfare and environmental friendly methods of production (Heaton et al. 2008). Resulting from this trend, local products around the world regained their fame and brought wealth to local producers, so product authenticity and authentication are emerging topics. Consumers in developed countries demand food products of high quality, one of the basic parameters being origin (Giovannucci et al. 2009).

A modern trend is the consumption of food produced locally, in order to reduce energy footprint and pollution through transportation. Determination of food authenticity is an important issue in quality control and food safety. In recent years, many serious diseases appeared related to foodstuffs, so providing the motivation for the scientific community to work more intensively in this area. Authenticity is a quality criterion for food and food ingredients and is required more and more worldwide, as a result of legislative protection for regional foods.

Foodstuff authentication may cover many different aspects, including mislabelling, misleading claims about origin and adulteration, which is defined as a process by which the quality or the nature of a given substance is reduced by adding a foreign or an inferior substance and removing a vital element or characterization of the product. European Union (EU) quality schemes identify products and foodstuff farmed and produced to exacting specifications. Product designations fall into two categories: those linked to a territory and those relating to a particular production method. Geographical indications and designations of origin are names identifying a product as originating in a given territory and testifying to a link between a given quality, reputation or characteristic of the product and its geographical origin. The EU regulation allows the application of the following geographical indications to a food product: (1) protected designation of origin (PDO), (2) protected geographical indication (PGI) and (3) traditional specialities guaranteed (TSG). PDO covers agricultural products that are produced, processed and prepared in a given geographical area using recognized know-how. PGI covers agricultural products and foodstuffs closely linked to the geographical area. At least one of the stages of production, processing or preparation takes place in the area. TSG highlights traditional character in composition or means of production.

Reviews of analytical methods for the determination of geographical origin of food and beverages have been published (Fairweather-Tait and Dainty 2002; Martinez et al. 2003; Carter et al. 2015; Li et al. 2016). Methods based on elemental composition have been reviewed as well as methods based on isotope ratios (Kelly et al. 2005; Carter et al. 2015; Li et al. 2016). Strategies employed to detect adulteration have relied on instrumental techniques and have evolved through time along with technology and instrumentation. High-performance liquid chromatography (HPLC), gas chromatography (GC), nuclear magnetic resonance (NMR), infrared spectroscopy (IR), fluorescence spectroscopy, capillary electrophoresis (CE) and more advanced techniques such as proton transfer reaction mass spectrometry (PTR-MS), electronic nose coupled with MS DNA technologies and sensory analysis have been proposed for food authentication (Martinez et al. 2003). However, organic components of a food crop production depend on various conditions (e.g. fertilization, history of the field, climatic conditions in the year of cultivation, geographic location and soil composition), so it is not always possible to determine the origin of a product by analysing the organic components. Despite uncertainty about the organic compounds in a sample, the content of selected elements (trace and rare earth elements, REEs) in food positively reflects the growing conditions in the particular environment. In addition, multi-isotope or single-isotope ratios (e.g. strontium and lead) can provide unique, representative fingerprints that make it possible to discriminate the origin of food samples. Over the past decade, with the development of new advanced analytical techniques [e.g. thermal ionization MS (TIMS), inductively coupled plasma MS (ICP-MS) and dynamic reaction cell-ICPMS (DRC-ICPMS)], we can successfully retrieve elemental and isotopic compositions, of any given food sample, and determine the geographic origin successfully. The growing concern of the consumers stimulated scientific research and publications in recent years, including multi-element and isotope ratio methods of analysis in food authentication after statistical evaluation of the data.

12.1.1 Basic Concept of Isotopes in Environment

Isotopes are atoms of a chemical element whose nucleus has the same atomic number, Z, but a different atomic mass, A. The word isotope means in the same place and comes from the fact that the isotopes are in the same place in the periodic table of the elements. The isotope ratio is the constant ratio of isotopes in chemical elements in nature and the characteristic size for each atomic species.

The atomic number corresponds to the number of protons in the atom. Therefore the isotopes of a given element contain the same number of protons. The difference in atomic masses stems from the difference in the number of neutrons in the atomic nucleus. In scientific nomenclature, isotopes are denoted by the names of a given element followed by a dash and the number of nucleons (protons and neutrons) in the atomic nucleus (helium-3, carbon-12, carbon-14, iron-57, uranium-238). In symbolic form, the number of nucleons is expressed above the chemical symbol on the left (^{3}He, ^{12}C, ^{14}C, ^{57}Fe, ^{238}U).

Isotopes of all elements form a set of nuclides. A nuclide is a definition for a particular atomic nucleus and is defined by the ordinal number Z and the mass number A: AZHh. Strictly speaking, it should be said that an element such as fluorine consists of one nuclide rather than one isotope. In a neutral atom, the number of electrons is equal to the number of protons. Therefore, the isotopes of a given element also have the same number of electrons and the same electronic structure. As the chemical behaviour of an atom is largely determined by its electronic structure, isotopes have almost identical chemical properties. The main exception is that, due to higher masses, heavier isotopes tend to react somewhat more slowly than lighter isotopes. This ‘mass effect’, or isotopic effect, is most pronounced for protium (1H) and deuterium (common name for 2H), because deuterium has twice the mass of protium. With heavier elements, the relative difference in mass is much smaller, so the effect of mass is usually negligible. Although isotopes have almost identical electronic and chemical properties, their nuclear properties differ dramatically. The atomic nucleus consists of protons and neutrons held together by a strong nuclear force. As the protons are positively charged, they repel each other. Neutrons that are electroneutral make a certain distance between protons, reduce electrostatic repulsion and stabilize the nucleus. For this reason, neutrons are needed to bind two or more protons to the nucleus. As the number of protons increases, additional neutrons are needed to form a stable nucleus, for example, although the neutron/proton ratio at ^{3}He is 1/2, the neutron/proton ratio at ^{238}U is >3/2. However, if too many neutrons are present, the nucleus becomes unstable. As the isotopes of a given element have different numbers of neutrons, they also have different neutron/proton ratios. This affects their nuclear stability, which results in the fact that some isotopes are subject to nuclear decay. The decay of these radioactive isotopes (shorter radioisotopes) is an important topic in nuclear physics. By studying the way this decay takes place, physicists gain insight into the properties of the atomic nucleus. Usually several isotopes of the same element can be found in nature. Stable isotopes are the most common, although significant amounts of unstable isotopes with long half-lives can be found, such as uranium-238. Small amounts of radioactive isotopes with a short half-life are also present in nature. These isotopes are formed as a product of the decay of larger radioactive nuclei with longer life. The atomic mass of an element in the periodic table of elements is the average of the natural distribution of isotopes of that element.

The natural distribution of various isotopes on Earth is ultimately the result of the amounts of isotopes formed in stars and supernovae, as well as the pattern of decay of radioactive nuclei formed in these processes. Then, the formation of the solar system also significantly affected the proportions of different isotopes that can be found on Earth, because solar winds could easily blow lighter nuclei towards further parts of the solar system immediately after the creation of the Sun. Because of this, the large gaseous planets are farther from the Sun.

12.1.2 Stable Isotopes

Most of the chemical elements in nature are mixtures of natural isotopes, which are either stable or radioactive (unstable). Eighty-one chemical elements have stable isotopes. These are all elements, from hydrogen ($Z = 1$) to bismuth ($Z = 83$), with the exception of technetium ($Z = 43$) and promethium ($Z = 61$). Many elements have more stable isotopes, which is why their relative atomic masses are not integers. Hydrogen is, for example, a mixture of stable isotopes ^{1}H and ^{2}H; carbon contains stable isotopes ^{12}C and ^{13}C; oxygen contains isotopes ^{16}O, ^{17}O and ^{18}O and so on. Tin has the most stable isotopes, 10 of them. In total, there are about 270 species of stable and about 70 species of radioactive nuclides in nature (Hölzl et al. 2004).

Radioactive isotopes, radioisotopes (radionuclides) of individual elements, are of natural or artificial origin. Many elements that contain stable isotopes have natural radioisotopes, as well as all-natural radioactive elements, that is, those that do not contain stable isotopes. These are the elements from polonium to plutonium ($Z = 84–94$) and the element promethium. Nuclear reactions can produce artificial radioisotopes of almost all elements, so today about 2500 types of nuclides are known, both natural and artificial. All of them can be classified in a nuclide map, in which each nuclide is represented by a square with different characteristic data (isotopic abundance, half-life, type and energy of radiation). The isotopes of the individual elements are arranged in horizontal rows according to the increasing mass.

12.1.3 Unstable Isotopes

Unstable isotopes are radioactive isotopes or radionuclides and are atoms that have a proton-neutron ratio greater or less than the ratio required for stability. Unstable isotopes tend to be stable, and this is achieved by radioactive decay (radioactivity). The consequences of radioactive decay are a change in the mass and/or chemical properties of radionuclides while emitting ionizing radiation. All the elements above the ordinal number 82 (lead) are unstable because the repulsive forces of the protons are no longer sufficient neutrons as equilibrium. The lead isotopes ^{206}Pb, ^{207}Pb and ^{208}Pb are the heaviest known stable atom, so lead is also the heaviest stable element of the periodic table. With the isotope 208Pb, the so-called double magic number is present in the nucleus. Lead isotopes are the end products of three of the four natural series of decays of radioactive elements. In this way, a relatively large amount of lead was formed, due to which there is a relatively large amount of lead in the Earth's crust in relation to other heavy metals (mercury, gold, etc.). Namely, the concentration of protons in one place is too high.

The nucleus of a radionuclide disintegrates spontaneously, passing into another nucleus. During decay, the nucleus of a radionuclide emits particles and/or electromagnetic rays of short wavelength. Emitted particles and electromagnetic rays are called ionizing radiation by one name. It is a phenomenon of radioactivity, and the process itself is transmutation, i.e. spontaneous transition from one nucleus to another, called radioactive decay.

A nuclide is an atom of a chemical element for which not only the ordinal or atomic number Z is accurately known but also the total number of nucleons (protons and neutrons) in the atomic nucleus. There are only 92 chemical elements in nature and another 12 artificially produced, and about 3100 different nuclides are known, of which 350 are natural and 2750 are artificial. Most nuclides are unstable (radioactive), about 2800. A suitable combination of protons and neutrons makes the atomic nucleus stable; as soon as it is different, the nucleus tends to approach a stable configuration by ejecting the nucleon. The energy levels in the core are replenished in order to reach the minimum energy configuration, that is, the maximum stability.

12.1.4 Isotope Applications in Analytics

There are several applications, which use the properties of different isotopes of a given element. One of the most common applications is a tracer or marker in a technique called isotope labelling (Kelly et al. 2005; Rossmann et al. 2000; Richards et al. 2003; Rhodes et al. 2010; Carter et al. 2015; Rees et al. 2016). Isotopes of a given element cannot be distinguished from each other by chemical methods. However, they can be distinguished by the use of physicochemical methods, such as mass spectrometry (direct differences in mass), infrared spectroscopy (based on vibrational frequencies in the molecule – heavier isotopes vibrate at lower frequencies than lighter ones), nuclear magnetic resonance, based on different gyromagnetic ratios, etc. Isotopic modification can also be used to determine the mechanisms of reaction through the kinetic isotope effect (Barling and Weis 2012). In addition to isotope labelling, several types of spectroscopy use the unique nuclear properties of specific isotopes. For example, nuclear magnetic resonance (NMR) spectroscopy can only be used for nonzero nuclear spin isotopes. The isotopes most commonly used in NMR spectroscopy are ^{1}H, ^{2}D, ^{13}C and ^{31}P. Mossbauer spectroscopy is based on the detection of nuclear transitions of certain isotopes.

The isotopic composition of the elements varies from planet to planet. This fact makes it possible to determine the origin of meteorites (Berglund and Wieser 2011). Some meteorites are classified as lunar or Mars meteorites. Radioactive isotopes also have important applications. The development of nuclear energy and nuclear weapons requires significantly higher amounts of certain isotopes (Rosman and Taylor 1998). The isotope separation process poses a significant technological challenge. Radioisotopes are also commonly used in medicine, biochemistry and chemistry as tracers. Small amounts of radioisotopes can be easily detected due to the characteristic emissions of the decaying nucleus (Berglund and Wieser 2011).

The natural radioactive decay of ^{14}C allows radiocarbon dating. Cosmic rays create the ^{14}C isotope in the atmosphere by erupting free neutrons from the air in a collision with nuclei. These neutrons can transform the nitrogen nucleus from nitrogen molecules (N_2) into the ^{14}C carbon isotope:

$$n + {}^{14}\mathrm{N} \rightarrow {}^{14}\mathrm{C} + \mathrm{p}$$

This isotope is eventually incorporated into the carbon dioxide molecule and thus reaches plants and through them animals. The isotope ^{14}C is, therefore, constantly formed in nature (due to cosmic rays) and decays (due to instability), and its concentration in living organisms is constant. However, in the inanimate world, there is no carbon dioxide exchange, and the ^{14}C concentration decreases with time. Thus, by measuring the residual radioactivity of ^{14}C in the biological sample, it is possible to determine the time when the carbon cycle was stopped (when the animal died, the tree was cut down, the plant was harvested, etc.).

12.1.5 Lead Isotope Ratios: Basics and It's Applications

Due to the complex environmental chemistry, transport patterns, meteorological conditions, the mixing of emissions from multiple sources and uncertainties associated with receptor modelling, it remains difficult to quantify the relative impact of emissions from different sources on metal accumulation in different foodstuffs. The emission and deposition of hazardous trace metals such as lead (Pb) is of significant concern because it can affect human and environmental health. Lead isotope ratio analysis is important as it is used for Pb-Pb dating in geochronology, and to trace the ratio, measurement can provide analytical information related to the source of lead contamination in naturally occurring samples (Lee et al. 2011). Studies of the isotopic composition of lead are therefore commonly used in the environmental, geological and anthropological studies (Krachler et al. 2004; Spiro, et al. 2004; Liu, et al. 2010; Lee et al. 2011; Marguí et al. 2010). Small Pb abundance variations occur in nature, and the isotopic composition of lead in the environment is dependent on the local pollutant source. If lead is present in the soil, a plant will take up small amounts, and subsequent isotope ratio studies might provide unique means of differentiating between different plant sources of origin. Local lead level may become mixed with external source of contamination that varies with time depending on the anthropogenic activity. There are four stable isotopes of Pb with the following approximate abundances: ^{208}Pb (52.4%), ^{207}Pb (24.1%), ^{206}Pb (22.1%) and ^{204}Pb (1.4%). ^{204}Pb is non-radiogenic and was not measured in this study because of its low relative abundance. ^{208}Pb, ^{207}Pb and ^{206}Pb are formed by the radioactive decay of ^{232}Th (half-life = 14 billion years), ^{235}U (half-life = 0.7 billion years) and ^{238}U (half-life = 4.5 billion years), respectively (Reimann and de Caritat 1998). Inductively coupled plasma mass spectrometry (ICP-MS) has been increasingly used in isotope ratio measurements in recent years. Lead isotope measurements provide analytical information relating to the source of lead contamination in naturally occurring samples (Reimann and de Caritat 2000). Concentration measurements cannot provide this kind of information. Studies of the isotopic composition of lead are therefore commonly used in environmental sciences and geological sciences as well.

12.2 Quality Impact of Food Authenticity: General Meaning

Food usually presents the area from which it originates and where it is produced, and in that way over time, it becomes a symbol of a certain locality, that is, it depicts the authenticity of a destination. That is why the concept of a geographical indication is most often associated with food. Therefore, for products that have a geographical indication, it can be said that this designation is a symbol of their diversity and it allows a clear boundary to be drawn between something that is quality and something that is less quality. As today’s consumer is increasingly demanding in terms of the value they receive for money, he also sets higher demands in terms of quality (Giovannucci et al. 2009). From the point of view of food, therefore, he insists on one that is of high and uniform quality. One of the aspects of food quality is its geographical origin, which consumers increasingly want to know about. Producers also realized the importance of this aspect of quality, and as a consequence of the interest of both consumers and producers, in the 1980s, the concept of geographical indication was developed, as part of the whole of intellectual property rights. There are over 10,000 products in the world that have a protected geographical origin. In the world, about 90% of products of protected geographical origin come from the member states of the Organization for Economic Co-operation and Development, and only 10% of products with a geographical indication come from developing countries (Giovannucci et al. 2009; Vandecandelaere et al. 2018). However, regardless of these data, it is interesting to note that some of the most recognizable products with geographical indications actually come from developing countries; examples are Mexican tequila, Colombian coffee, Darjeeling tea from the Himalayas and basmati rice from India (Vandecandelaere et al. 2018). As many as 6000 products with a geographical indication originate from the member states of the European Union, and among them, in terms of the number of protected products, Italy leads, followed by France, Spain, Portugal and Greece. The largest number of protected products is agricultural and food products such as meat, cheese, cereals, fruits, vegetables and oils (Giovannucci et al. 2009).

The importance of food with a geographical indication is seen through several positive influences on economic aspects. First of all, it was determined that products with geographical indications have higher prices (by an average of 20–50%), as given by Vandecandelaere et al. (2018). The higher price of these products comes from the costs that arise during the registration and certification procedures. Since the concept of the geographical indication is such as to protect producers from unfair competition, it is clear that those who have invested in the process of protecting the origin of their product can expect higher earnings, which are returned to them through the placement of these products. On the other hand, this way of doing business, when producers see all the benefits and embark on the registration process, reduces the number of those who operate within the 'grey economy', because the registration and certification process redirect them to legal flows, which undoubtedly have significance for the state budget. The export of protected products to the world market has been facilitated because the labels are recognizable everywhere in the world. Then, in the long run, there is a positive impact on the volume of production, because due to the increase in demand and market expansion, the production of a particular product increases, in order to meet the demands of the market (Vandecandelaere et al. 2018). The protection of products by geographical indications of origin avoids the effect of market price volatility because primary producers, thanks to the minimum agreed price within their associations, are protected from these fluctuations. Then, the impact on the economy is reflected in the fact that certain products that are not protected by geographical indications and originate from the same geographical area in which the product of the same category, but protected geographical origin, increase its price based on the reputation of the product label (Vandecandelaere et al. 2018). All this together has an impact on accelerating production in a certain region, which certainly has an impact on the local development of underdeveloped areas. In this way, new jobs are created at a certain destination, the number of employees increases, and at the same time, all this represents a potential chance for the development of tourism.

12.2.1 Food Authenticity

The authenticity or originality of a food means the authenticity of all the information highlighted on it, from the name of the food, the list of ingredients, the net amount of filling or the origin of the food. By using a food name defined by regulations (e.g. liver pate, rye bread or butter), the manufacturer undertakes to ensure that his product meets the standard quality criteria prescribed for that name. Thus, liver pate must, inter alia, contain ≥10% liver, rye bread must be produced from ≥70% rye flour, and butter must have ≤16% water, in order to be authentic (Vandecandelaere et al. 2018). Deviations from such limit values are considered non-compliances with food regulations, but, in the case of proven intent, they can also be considered counterfeiting, i.e. frauds that unjustifiably gain material benefit. In a narrower sense, food counterfeiting involves the following types of actions: (a) the use of raw materials or ingredients that are of lower quality (and therefore lower price) than standard or declared, (b) sale of products under a false name and (c) the use of ingredients intended to mask the poor quality of the product. Certain types of food, despite their authenticity, can be replaced with similar foods and thus reduce the value of the market price. Counterfeiting of food, in such a way that its composition deviates from the standard composition that can be expected with regard to the name of the food or the declared values, deprives the consumer not only in financial but also in health and spiritual terms. Namely, in the long run, such adulteration of food can have a negative impact on the health of the population, because such food calls into question the balance of the diet and the acceptability of nutritional profiles. On the other hand, food is associated in many religions with the realization of spirituality, and religious affiliation often includes permanent or occasional dietary restrictions in terms of the choice of type of food and the manner of its preparation.

12.2.2 ‘Fingerprinting’ of Food Products

Food ‘fingerprinting’ is the process of identifying a unique isotopic imprint of an ingredient using isotope ratio mass spectrometry (IRMS). This imprint consists of the relationship to the met key elements (carbon, nitrogen, oxygen, hydrogen and sulphur), because each of them is influenced by the source material, production process and environmental conditions specific to the place of origin. Therefore, it is unique for each ingredient and can be used to determine where it was produced and to which it was subjected. Taking a ‘footprint’ of food can be used for confirmation of the geographical origin of the ingredient; examining the authenticity of ingredients listed as natural; identification of undeclared additives, such as sugar; and detection of the use of synthetic fertilizers in organic products. Thus way, it can help to prevent food fraud, reduce risks, maintain full control of the supply chain, strengthen your reputation and increase customer loyalty.

12.3 Analytical Methods

12.3.1 Trace and Rare Earth Element Analysis

The techniques used for the elemental fingerprinting of plant species, food and beverages are mostly those with multi-element detection capability: inductively coupled plasma with atomic emission spectrometry (ICP-AES) and inductively coupled plasma with mass spectrometry (ICP-MS); ICP coupled to atomic emission spectroscopy (ICP-AES) provides high reproducibility and a quantitative linear range greater than conventional methods with arc or spark, allowing the simultaneous determination of metals and non-metals. ICP-AES uses a very high-temperature excitation source and molecular interferences are greatly reduced. However, analysis is more expensive and all samples have to be introduced in a dissolved form. Furthermore, in ICP-AES, complex samples usually provide patterns that are very difficult to interpret. ICP-AES is also a fast, multi-element technique with an extended dynamic linear range and moderate-to-low limits of detection (LODs). Up to 60 elements can be screened per sample run in less than 1 min. ICP-AES monitors wavelengths down to 165 nm that provide high sensitivity for phosphorous and sulphur determinations.

ICP-MS is a powerful tool for elemental analysis, providing LODs for more than 70 elements at low concentrations, typically parts per billion or trillion (ppb or ppt). Conventional ICP-MS systems cannot provide the lowest LODs, because of the polyatomic interferences that can enhance the background signal or overlap the signals of the most abundant isotopes. The use of a DRC can remove unwanted interferences by creating specific chemical reactions with a supplementary gas, which improves the selectivity and the sensitivity dramatically.

12.3.2 Multi-isotope Ratio

Investigations of the ratios of stable isotopes and the contents of unstable isotopes (radioisotopes), especially heavy elements (e.g. uranium, strontium, thorium or lead), have been used primarily in geological sciences for age determination (Webster and Oliver 2001; Taylor et al. 2015). For heavier elements (e.g. Pb and Sr), fractionation is insignificant compared to the original isotopic ratios because the nuclides have high mass compared to the mass differences of the isotopes. In this case, stable isotopic ratios depend essentially upon the origin of the ore body and can be used for identification of the source of a material or characterization of its transportation history (e.g. $^{208}Pb/^{207}Pb$). Strontium is the commonly used heavy metal for isotope ratio analysis. Clearly, the present $^{87}Sr/^{86}Sr$ ratio is greater for samples that have greater ages, *t*, and samples with larger Rb/Sr ratios. The $^{87}Sr/^{86}Sr$ ratio therefore varies between different rock types and formations. Since Rb is an alkali metal and Sr is an alkaline earth metal, these elements behave differently in geological processes, creating large variations in Rb/Sr and, so, large variations in $^{87}Sr/^{86}Sr$. The $^{87}Sr/^{86}Sr$ ratio has been shown to vary widely in surface rocks, so any Sr released into soils, rivers and groundwaters has an isotopic signature that reflects its source. Sr isotopes have also been used to trace agricultural products, which have incorporated Sr, along with Ca, from soils incorporating the Sr isotope ratios of the underlying rocks.

12.3.3 Lead Isotope Ratios: Measurement Challenges

Whichever methodology is selected, the natural or ‘common lead’ ^{204}Pb isotope is measured as a reference to calculate the original (primordial) level of the other (mainly radiogenic) Pb isotopes, so accurate measurement of ^{204}Pb is essential (Taylor et al. 2015). Unfortunately, for measurements made using inductively coupled plasma mass spectrometry (ICP-MS), ^{204}Pb suffers an isobaric interference from ^{204}Hg (an isobaric overlap is where isotopes of different elements occur at the same nominal mass) meaning that any mercury present as a contamination or as a component of the sample would bias the measurement of ^{204}Pb.

12.4 Chemometric Tools

12.4.1 Multivariate Analysis

Multivariate statistical analysis (MSA) has been present in science for almost a century. However, its application in the natural sciences began only in the late 50s of the 20th century. Over time, multivariate analysis applications have become more common as they have been increasingly appreciated by both scientists and business people. Before the advent of MSA, most studies used analyses that processed a maximum of two variables simultaneously. This analysis is based on measures of central tendency (arithmetic mean and median), measures of variation (variance, standard deviation and quarters), confidence intervals and tests based on normal distribution, t-distribution, etc. The farthest reach in the study of the relationship between the two phenomena was the correlation coefficient. MSA has provided much more powerful techniques that have allowed researchers to discover patterns of behaviour in the interrelationship of a large number of variables, patterns that would otherwise be hidden or barely noticeable. In addition, most techniques are precise enough to use a statistical significance test to determine whether a particular interdependence is really relevant or whether it is the result of data fluctuations in sample. These techniques have significantly increased the amount of usable information that can be extracted from the observed statistical data. Today, with the help of high-tech computers and available statistical software, the results of multivariate statistical analysis take only a few seconds. A variable or variable in the context of MSA is any phenomenon that varies freely in such a way that these variations can be identified and measured. When MSA is applied in research, we come across an inexhaustible set of phenomena that can be analysed, such as, for example, imports, exports, sales, costs, customer preferences, social product, national income, etc.

A multivariate technique can analyse the interrelationships between several variables (more than two), simultaneously according to the appropriate model on which the technique is based. Most techniques identify patterns (patterns) of concordance or relationships between many variables, assess the relative importance of each variable and predict or explain possible outcomes. All multivariate statistical techniques can be divided into two groups:

- Techniques that analyse addiction
- Techniques that analyse interdependence

In dependency analysis techniques, the goal is to explain one dependent variable and predict its variation based on other independent variables.

There is often a need for a combination of techniques from the two groups. All multivariate techniques have been developed based on research by experts from various scientific disciplines. As a result, multivariate statistical analysis is interdisciplinary both in its origin and in its application. The techniques presented in this book are those that are currently most used in research. Many of these techniques have a strict mathematical derivation and basis and belong to 'classical' statistical models. Other techniques include various approximation methods and solutions that have proven to be sufficiently accurate and usable through practice.

Rank correlation is when the original values of a feature are replaced by ranks. If it is necessary to observe the correlation of phenomena whose characteristics cannot be measured numerically or if it is necessary to quickly reach at least the approximate value of the correlation coefficient, Spearman's (for two phenomena) and Kendall's rank correlation coefficient (for more than two phenomena) are most commonly used.

Multiple linear regression predicts the dependence of one phenomenon on two or more independent phenomena and is examined; then we are talking about multiple or multiple regression. The task of regression is to discover as many factors as possible. The starting point is the assumption: the more independent phenomena in the model, the smaller the latent influence variables (random errors).

12.4.2 Correlations and Regression Analysis

Correlation is the interrelationship between different phenomena that are represented by values in two or more random variables. In this case, the connection means that based on the knowledge of the value of one variable, with a certain probability, it is possible to predict the value of another variable, since these values appear in a certain ratio. The degree to which two values coincide can be represented graphically on a scatter plot or by a correlation coefficient.

Regression analysis is an extension of correlation analysis and is one of the most commonly used statistical techniques today. Regression analysis is a set of analytical techniques used to better understand the interrelationship between the phenomena observed, expressed in the form of collected data. As an end result, the analysis produces a regression equation, but all the results obtained in this process can provide valuable information about the observed phenomena and their environment. Basically, regression analysis involves two or more variables that are related to each other in some way. One of the variables is of special interest, because the purpose of the analysis is to explain the changes in it through changes that occur simultaneously on other variables in the model. This variable is called the dependent or criterion variable and is denoted by Y. Other variables are used to explain or predict the value of the dependent variable. These variables are called independent or predictor variables and are denoted by X. Usually more independent variables are used in the model, so they are denoted by X_1, X_2, … X_n, where n denotes the total number of independent variables in the regression model. Predictor variables are also called independent, covariates, regressors, factors or carriers. Although most commonly used, the name of the independent variable is the least appropriate to reality, as these variables are rarely independent of each other. The relationship between dependent and independent variables is expressed in the form of one or more equations called a regression model. The actual relationship between the dependent and independent variables can be approximated by the following regression model:

$$Y = f\left(X_1, X_2, \ldots X_p\right) + \varepsilon,$$

where ε is a random error that represents the difference between the approximation and the actual value of the dependent variable Y and the function of describes the relation between the dependent and independent variables. Regression models can be divided according to several criteria. Below are some of the divisions. According to the number of independent variables in the regression model, there is a simple regression, in which there is one dependent and one independent variable, and multiple regression, where there is one dependent but several independent variables. According to the type of dependent variable, regression models can be divided into:

- Models with a continuous dependent variable
- Models with a categorical dependent variable that is not dichotomous but takes more than two values (categories)
- Models with a dichotomous dependent variable that represents a special case of models with a categorical dependent variable, because the dependent variable can take only two values.

According to the type of relationship between dependent and independent variables, regression can be:

- Linear regression, which is characterized by the existence of a linear relationship between independent variables and the dependent variable and which is expressed in the model as the addition of independent variables of the first degree
- Nonlinear regression, which can be:
- Quadratic regression
- Polynomial regression
- Exponential regression, etc.

According to the number of dependent variables, the regression model can be:

- Univariate regression model, i.e. model with one dependent variable

- A multivariate regression model in which more dependent variables occur, which is why it consists of several regression equations

In addition to enabling the assessment of the degree of correlation of phenomena, regression analysis also gives the probability of occurrence of the calculated correlation. It is used to test hypotheses in studies and experiments but also to predict the future movement of the observed phenomenon. The concept of regression is easy to understand and is implemented in almost every statistical package and allows the examination of the functional dependence between variables, so as such it is the basis of many modern statistical techniques.

12.4.3 Cluster Analysis

Cluster analysis is a statistical technique for determining relatively homogeneous groups of objects. It is used in various branches of science to categorize or classify individual units of analysis (objects or respondents) with regard to their similarity or difference according to some of their measured characteristics. Although both discriminant analysis and cluster analysis relate to the problem of classifying objects or respondents into categories, discriminant analysis requires knowledge of group affiliation for the units we use to determine classification rules. For example, if we try to distinguish subjects who fall into three diagnostic categories, we must know the diagnosis (group affiliation) for each subject. In the cluster analysis, the group affiliation of the objects is not known, nor is the final number of groups. The goal of cluster analysis is to identify homogeneous groups or clusters.

12.5 Case Study of Isotopic Ratio Measurements

The aim of this case study was to evaluate lead composition of red goji berry (*Lycium barbarum* L.) cultivated in the Republic of North Macedonia compared to the same species obtained in market in China. Several metrics have been applied for authenticity testing of the samples: total lead content, multi-elements' characterization with clustering and lead isotopic ratios.

12.5.1 Instrumental Conditions for Isotopic Measurement

To confirm that Pb does not undergo a charge-transfer reaction (and to check if any cluster ions are formed), 20 μg/L Pb solution was aspirated, and a mass scan was performed. The sum of the signal for these clusters amounted to <0.5% of the total Pb signal (the sum of the unreacted precursor and reacted product Pb ions); at this level the product ions can be considered negligible and ignored (Fig. 12.1). Pb did not appear to undergo any charge transfer reaction, as the Pb signal with argon gas in the cell remained at practically the same level as with no reaction gas. Very good sensitivity was obtained for ^{206}Pb, ^{207}Pb and ^{208}Pb isotope concentration measurements (Fig. 12.2). Satisfactory linearity (R) was obtained in the range from 5 to100 µg/L (Fig. 12.2). The instrumental detection limits for the three isotopes (^{206}Pb, ^{207}Pb and ^{208}Pb) were calculated as 0.35, 2.86 and 0.85 µg/L, respectively.

12.5.2 Model Testing and Implementation

The samples were prepared according to a validated procedure for multi-element chemical analysis. ICP-MS optimization was performed using a standard tuning solution (containing 1 µg/L each of Li, Mg, Y, Ce, Tl and Co in a 2% HNO_3 matrix). The multi-element analysis included quantification of the isotopes and is given in Table 12.1. Special emphasis has been given to the lead content in the goji berry samples (Table 12.2). Comparative analysis has been applied between the two groups of samples (geographical regions), where the significant difference has been evaluated for the total Pb content in samples. The same occurrence has been evident when the isotope ratios were calculated (Table 12.3, Fig. 12.3).

12.5.3 Metrics Tools

Multi-element analysis covered the characterization of more than 30 elements, as given in Table 12.1. Comparative analysis for each individual element could not provide unified information. As multi-elemental analysis involves the analysis of essential nutrients, nutrients and trace elements, its content in the samples may vary independently of the geographical area. But on the other hand, this data, if pro-

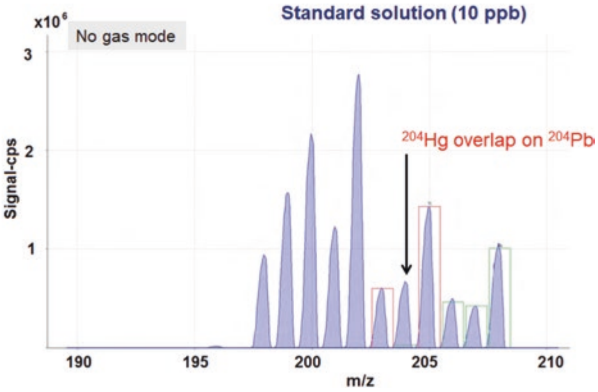


Fig. 12.1 Mass scan with ICP-MS, sensitivity for Pb

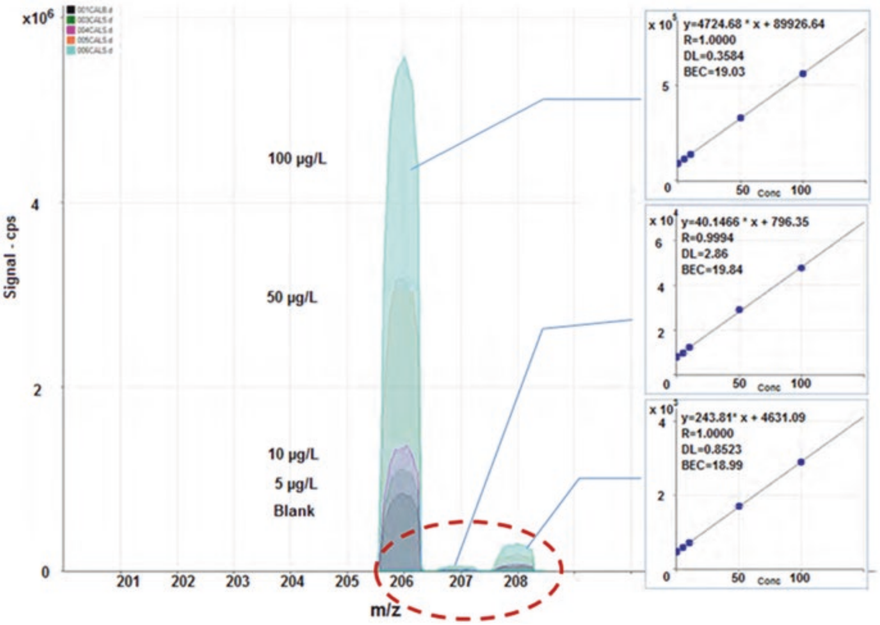


Fig. 12.2 Calibration data for ^{206}Pb, ^{207}Pb and ^{208}Pb isotopes

cessed with certain statistical tools, can provide identification data. Therefore, after the chemical characterization of the samples, the obtained information on the contents of the elements is normalized by logarithmic transformation, and component analysis and cluster analysis are applied. In both cases there is a clear identification of the two groups depending on the geographical area (Figs. 12.4 and 12.5). Thus, the model proved to be a successful and useful tool in the process of determining/confirming the geographical origin of the samples.

12.6 General Conclusions and Perspectives

Determination of the geographical origin of food and beverages has been a growing issue over the past decade for all countries around the world, mostly because of the concern of consumers about the authenticity of the food that they eat. An increasing number of research articles in the past 5 years have investigated the elemental composition and the isotope ratios as indicators to determine the origin of food and beverages. The proposed investigation will be the first attempt of application of a validated method including multi-element and multi-isotope chemical characterization and the statistical tools in order to use for determination of the geographical origin of food and beverages. Comparative analysis can help in improving the large-scale application. Isotope ratio mass spectrometry is a promising tool for origin

Table 12.1 Data summary of the instrumental condition and validation of the applied methodology

Element	Isotope	ORS mode	Integration time (*s*)	Linear range (µg/L)	Slope	Intercept	*R*	LOD (µg/L)	LOQ (µg/L)
Li	7	Normal	0.1	0.05–10	0.045	0.023	0.9997	0.042	0.140
Be	9	Normal	0.1	0.05–10	0.010	0.166	0.9960	0.012	0.040
B	11	Normal	0.1	5–50	0.295	−7.648	0.9989	0.98	3.273
Na	23	Normal	0.1	30–1000	2.125	391.2	0.9992	8.12	27.12
Mg	24	Normal	0.1	20–1000	1240	13785	0.9995	5.77	19.27
Al	27	Normal	0.1	5–100	0.882	19.36	0.9990	1.22	4.075
P	31	Helium	0.5	20–1000	13455	12963	0.9991	5.96	19.91
K	39	Normal	0.1	30–1000	35964	40552	0.9995	10.2	34.07
Ca	42	Normal	0.1	10–1000	1796	3995	0.9991	2.34	7.816
Ti	48	Helium	0.5	0.5–10	1.36	5.998	0.9996	0.12	0.401
V	51	Normal	0.1	0,5–10	0.022	0.799	0.9999	0.096	0.321
Cr	53	Helium	0.5	0.055–10	0.024	−0.051	0.9991	0.0063	0.021
Mn	55	Normal	0.1	0.2–10	1.754	−9.321	0.9993	0.074	0.247
Fe	56	Helium	0.5	0.1–100	0.55	1.90	1.0000	0.017	0.057
Co	59	Normal	0.1	0.01–10	1.528	−0.724	0.9996	0.0031	0.010
Ni	60	Normal	0.1	0.015–50	0.042	1.33	0.9993	0.0044	0.015
Cu	63	Helium	0.5	0.5–10	0.66	10.24	0.9991	0.199	0.665
Zn	64	Helium	0.5	0.01–10	0.322	7.82	0.9995	0.0017	0.006
Ga	69	Normal	0.1	0.05–10	0.037	−0.0059	0.9994	0.013	0.043
Ge	72	Normal	0.1	0.015–10	0.0014	0.0055	0.9990	0.0046	0.015
As	75	Helium	0.5	0.005–10	0.010	0.0022	0.9993	0.0011	0.004
Se	77	Helium	0.5	0.01–10	0.0069	−0.0037	0.9997	0.0019	0.006
Rb	85	Normal	0.1	0,5–10	1.150	2.483	1.0000	0.10	0.334
Sr	88	Normal	0.1	2–100	1.680	10.27	0.9995	0.77	2.572
Mo	95	Normal	0.1	0.01–5	0.0079	0.0022	0.9991	0.0033	0.011
Ag	107	Normal	0.1	0.05–5	0.0037	−0.0059	0.9995	0.012	0.040
Cd	111	Normal	0.1	0.01–5	0.021	0.0025	1.0000	0.0044	0.015
Sn	118	Normal	0.1	0.01–5	0.0011	0.00069	0.9994	0.0012	0.004
Sb	121	Helium	0.5	0.1–10	0.038	10.47	0.9998	0.054	0.180
Cs	133	Normal	0.1	0.5–5	0.144	18.59	0.9991	0.13	0.434
Ba	137	Normal	0.1	1–10	0.058	−0.0063	0.9994	0.29	0.969
Tl	205	Normal	0.1	0.2–5	2.019	−5.94	1.0000	0.062	0.207
Pb	206/207/208	Normal	0.1	0.1–10	0.023	0.0647	0.9991	0.019	0.063
Bi	209	Helium	0.5	0.1–5	0.0092	−0.1046	0.9992	0.54	1.804

ORS octopole reaction system (normal, Ar gas is applied)

Table 12.2 Total content of lead in goji samples – comparative analysis

	Samples from China (in mg/kg)				Samples from North Macedonia (in mg/kg)					
Element	min	max	Mean	SD	min	max	Mean	SD	*t*	p^a
Pb	0.015	0.096	0.054	0.028	0.008	0.028	0.022	0.01	3.08	0.03

[a] Significant difference occurs for obtained values of $p < 0.05$

Table 12.3 Data summary for the Pb isotope ratios (comparative analysis)

Sample	N	$^{207}Pb/^{206}Pb$	$^{208}Pb/^{206}Pb$
Goji berries – North Macedonia	10	0.951 ± 0.15	1.062 ± 0.09
Goji berries – China	10	0.868 ± 0.10	2.070 ± 0.08

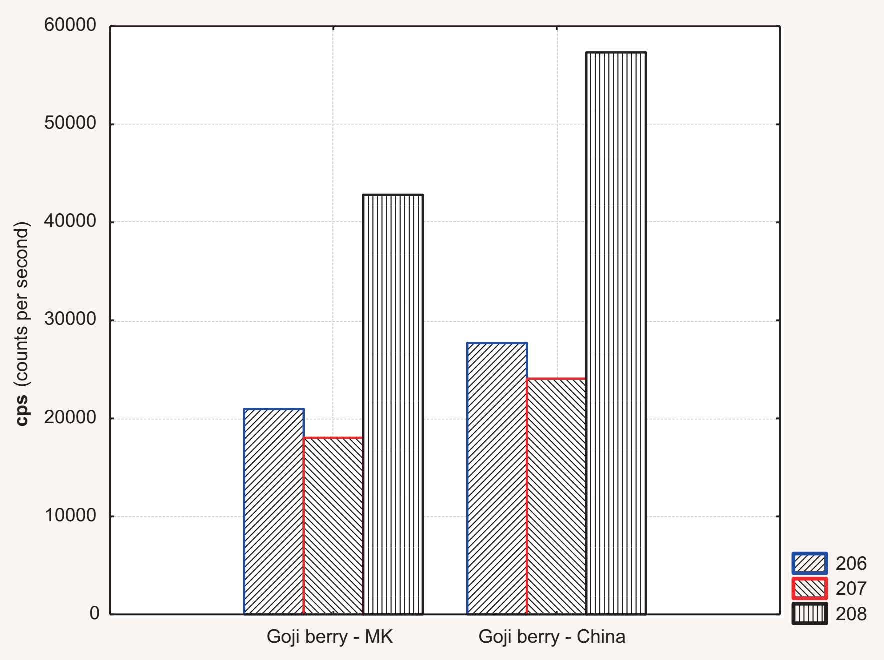


Fig. 12.3 ICP-MS intensity measurements (cps) for ^{206}Pb, ^{207}Pb and ^{208}Pb isotopes

assignation of food samples. The technique is sometimes able to distinguish the geographical origin of samples by itself. More frequently it is very effective to use it with other determinations and then combined via multivariate statistics. One of the greatest limitations to the application of the technique in the origin assignation is the lack of large databases of isotopic abundances in food items.

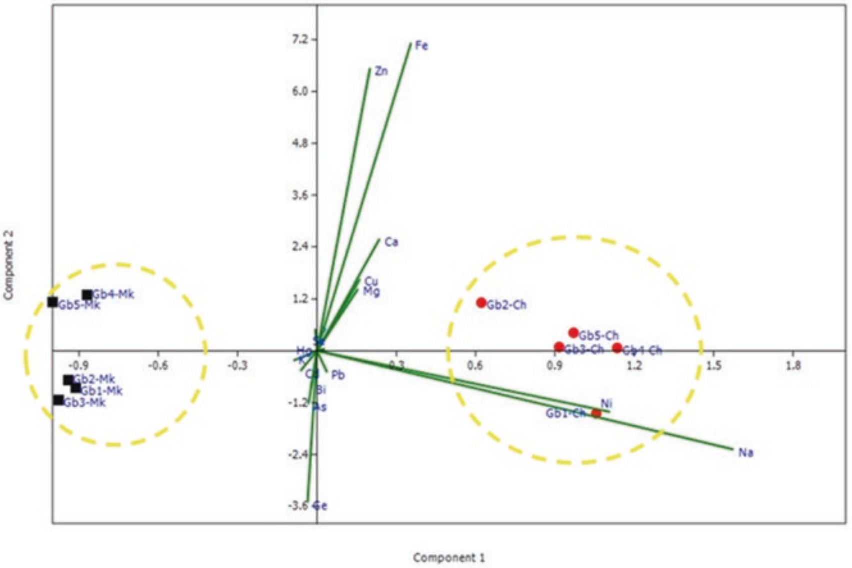
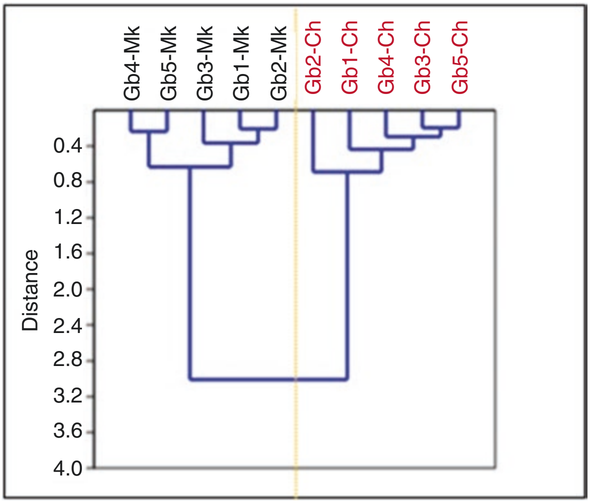


Fig. 12.4 Principal component analysis of multi-element data for analysed samples (Ch Chinese samples, MK Macedonian samples)

Fig. 12.5 Cluster analysis of multi-element data for analysed samples (Ch Chinese samples, MK Macedonian samples)



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Chapter 13
Accumulation Abilities of Endemic Plant Species from the Vicinity of an As-Sb-Tl Abandoned Mine, Allchar, Kožuf Mountain

Katerina Bačeva Andonovska, Trajče Stafilov, and Vlado Matevski

Abstract The Allchar mine, located in the southern part of the Republic of Northern Macedonia, has a unique mineral composition. This mine locality is world-famous for its thallium minerals but also known for the large amounts of minerals of arsenic and antimony. Because of the specificity and enriched contents of these potentially toxic elements in the soil, at this site grow seven local endemic plant species: *Viola allchariensis* G. Beck, *Viola arsenica* G. Beck, *Thymus alsarensis* Ronn., *Centaurea leucomalla* Bornm., *Onobrychis degenii* Dörfler, *Knautia caroli-rechingeri* Micev., and *Centaurea kavadarensis* Micev. The main goal of this investigation was to establish the accumulation of some toxic elements (As, Sb, Tl) in relation to its mobility in the endemic plant species. Investigations have been initiated to determine the levels of uptake and distribution of As, Sb, and Tl, as well as some other toxic elements, to the different parts of plant tissues (root, stems, leaves, flower, and seeds) of these endemic species. The plant samples were digested and then analyzed, applying atomic emission spectrometry with inductively coupled plasma (ICP-AES). It was found that the accumulation of As, Sb, and Tl in these endemic

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species is significantly high. Such behavior of As, Sb, and Tl was confirmed by the extraction tests of soil samples applying various solvents.

Keywords Alchar mine · Endemic plant species · Boacumulation · Arsenic · Antimony · Thalium

13.1 Introduction

Contamination of soils with toxic elements represents a serious threat to environmental quality and human health. Although toxic elements can be incorporated into soils from natural sources (weathering of parent materials, emissions from volcanoes, mining, industrial pollution, etc.), most of their inputs have an anthropogenic origin (Alloway 1995).

Soil toxicity has become a global concern in different parts of the world, with the increasing contamination of soil, water, and plants in many regions of the world. The intense industrialization and mining activities that were developed over the years were carried out without pollution control, in accordance with the absence of an environmental legislation. The environmental risk of the toxic metal residues, chaotically abandoned in the environment over the years, was affected by runoff waters, and they depend on the stability of the metal-bearing ores. Some of the plant species that grow on such land respond differently depending of their ability to uptake or exclude a variety of elements in their shoots during normal growth and reproduction.

Certain plant species can accumulate essential and nonessential heavy metals in their roots and shoots to levels far exceeding than those present in the soil. Metal-accumulating plant species are always restricted to metalliferous soils found in different regions around the world. The mechanisms of metal accumulation (from soil to plant species), which involve extracellular and intracellular metal chelation, precipitation, and translocation in the vascular system, are poorly understood. Interest in these mechanisms has led to the development of phytoremediation, a new technology that involves the use of plants to clean up polluted soil with toxic elements. Medicinal plants can absorb environmental contaminants from the ground, which may consequently lead to harmful effects on human health when they are taken. In addition, there is a great need to control the level of pollutants in herbs, with particular attention to the level of toxic metals (Tripathi et al. 2012). As such, detailed studies of soils and vegetation at such strongly metal-contaminated sites and their peripheries are necessary to accurately assess implications for toxicity to herbivorous consumers and impact on general human health. Such studies are also essential for gathering valuable information for reasoning strategies of phytoremediation and land reclamation in metalliferous sites (Peer et al. 2006; Jimenez et al. 2013).

The physiological mechanisms of tolerance have been categorized as either exclusion, blocking the movement of metals at the soil/root or root/shoot interface, or accumulation, allowing uptake of metals into aerial parts and rendering them nontoxic through chemical binding or intracellular sequestration (Baker 1981; Baker and Walker 1990; Ernst et al. 1992). Combinations of all of these mechanisms in the plant species contribute to metal tolerance or hyperaccumulation. Additionally, in most plant species, metals are predominantly accumulated in the roots. As a consequence, the ratio shoot/root of metal concentrations (the so-called translocation factor, TF) is generally substantially below unity in these plants. Hyperaccumulation can be seen as a special and extreme case of the wider category of accumulators where metal concentrations are much higher in the leaves than in the roots, with TF abnormally high and above unity (Krämer 2010).

Only a restricted number of plant species from the local flora are able to grow in metalliferous soils. In several studies, these plants are shown to have physiological adaptations, such as metal tolerance or metal accumulation, that are distinct from non-tolerant plant species of the same species that grow in uncontaminated soils, supporting the idea of different ecotypes (Lefebvre and Vernet 1990). Reclamation of metal-contaminated sites by plant species native to toxic areas leads to stabilization of the soil, immobilizing trace elements in the rhizosphere and, thereby, reducing the risk of dissemination of metalliferous dust by wind or water erosion.

Understanding the metal accumulation physiology of the plants through the metal contents in the different plant components is important. Studies have shown that metal ions from soil are absorbed by the root system and transported to the stem tissues through the xylem prior to their sequestration in leaf tissue (Hall 2002; Harper et al. 2002). These investigations show that such areas are characteristically different specifically with the underlying geology and the associated type of soil formation. With vegetation largely influenced by soil type, it can be inferred that unique plant species occur in such area.

Among the most toxic elements emitted by mining and mining activities are antimony, arsenic, and lead. These elements are frequently present together in ore minerals or in mineral associations. The exploitation of such deposits for the economic ore minerals provokes a simultaneous release of such toxic elements into the environment. None of them has a known biological function in animals or plants, being highly toxic, especially to mammals. Inorganic arsenic species are generally highly toxic to plant species. In humans arsenic and antimony cause a great variety of adverse health effects, including mutagenic and carcinogenic effects, whereas Pb mainly causes hematological, gastrointestinal, and neurological dysfunctions, with children being particularly sensible (Winship 1987; Lockitch 1993; Mandal and Suzuki 2002; Sundar and Chakravarty 2010). Arsenate acts as a phosphate analog and affects phosphate metabolism, while arsenite reacts with sulfhydryl groups of enzymes and tissue proteins, leading to inhibition of cellular function and death (Meharg and Hartley-Whitaker 2002).

In arsenic non-hyperaccumulating plants, resistance to environmental arsenic has been shown to involve a decreased uptake of arsenate due to suppression of the high-affinity phosphate uptake system (Meharg and Macnair 1991, 1992). The study of the environmental status of this kind of scenarios is, therefore, crucial to establish the current and eventual environmental and health impacts and to assess the right measures to be undertaken in order to minimize such impacts.

So far, many studies have been carried out for the extraction and accumulation of toxic elements in many plants (Baroni et al. 2004; Antosiewicz 2004; Chang et al. 2005). The extraction of toxic elements from the soil by the plant still depends on the presence and the solubility of the elements in the soil where the plant grows and develops, the type of soil, the physical-chemical properties of the soil, the type of plants species, the age of the plants, and the exposure time of these plants on such soils (Marin et al. 1992).

The main goal of this study was (i) to establish the accumulation of some toxic elements (As, Sb, Tl) to the different parts of plant tissues, in relation to its mobility in the endemic plant species; (ii) to carry out the environmental characterization of the soils impacted by the former exploitation of As-Sb-Tl deposit "Allchar," Republic of North Macedonia; and iii) to study the seven local endemic plant species *Viola allchariensis* G. Beck, *Viola arsenica* G. Beck, *Thymus alsarensis* Ronn. (Bačeva et al. 2016), *Centaurea leucomalla* Bornm. (Bačeva et al. 2018a), *Onobrychis degenii* Dörfler, *Knautia caroli-rechingeri* Micev., and *Centaurea kavadarensis* Micev. (Bačeva et al. 2018b) and one subendemic species – *Viola macedonica* Boiss. and Heldr. (Bačeva et al. 2014a), which grow spontaneously in this area.

13.2 Material and Methods

13.2.1 Study Area

The locality of Allchar is volcanogenic hydrothermal mineral deposit and is situated at the northwestern part of the Kožuf Mts. (Republic of North Macedonia), close to the border between the Republic of North Macedonia and Greece (Fig. 13.1). From the geological and geotectonic point of view, ore mineralization is related to a Pliocene volcano-intrusive complex (Janković et al. 1997; Volkov et al. 2006; Boev and Jelenkovic 2012; Amthauer et al. 2012). The Allchar locality contains several geological formations: complex of Precambrian metamorphic rocks; complex of Mesozoic rocks; complex of Upper Cretaceous sediment rocks; complex of Pliocene sediments, pyroclastics, and volcanic rocks; and complex of Quaternary sediments. Several ore bodies occur within a zone of 2 km in length and around 300–500 m wide. The most important ore minerals are Fe sulfides, As and Tl minerals, cinnabarite, and As, Pb, and Sb sulfosalts (Balić Žunić et al. 1993; Boev et al. 1993, 2001–2002; Volkov et al. 2006; Jelenković and Boev 2011). As a result of mineralization, the entire area of a few square kilometers is enriched with toxic elements such as arsenic, thallium, and antimony.

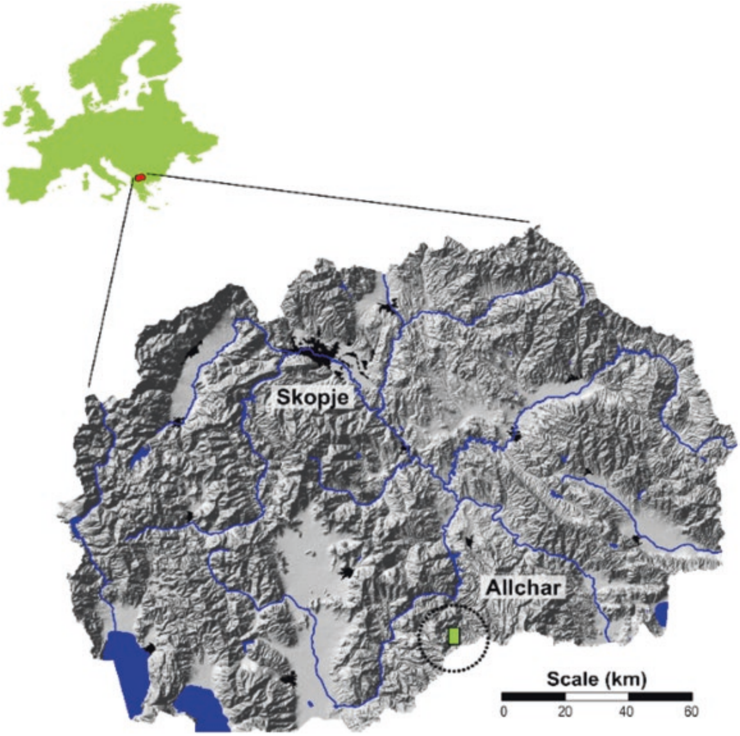


Fig. 13.1 The location of the Allchar mine

13.2.2 Study Species

Seven local endemic and one subendemic plant species can be found on the locality of Allchar, Republic of North Macedonia. The local endemic plant species that grow on these soils are *Viola allchariensis* G. Beck, *Viola arsenica* G. Beck, *Thymus alsarensis* Ronn. (Bačeva et al. 2016), *Centaurea leucomalla* Bornm. (Bačeva et al. 2018a), *Onobrychis degenii* Dörfler, *Knautia caroli-rechingeri* Micev., and *Centaurea kavadarensis* Micev. (Bačeva et al. 2018b), while *Viola macedonica* Boiss. and Heldr. is a subendemic plant species with a wider distribution on the Balkan Peninsula (Bačeva et al. 2014a). The results showed that all studied endemic species are able to hyperaccumulate toxic elements in their roots, stems, leaves, flowers, and seeds.

Three species of the genus *Viola* grow at the same site, around the abandoned As-Sb-Tl mine of Allchar: *Viola arsenica*, *Viola allchariensis*, and *Viola macedonica* (Fig. 13.2). All species belong to the Section *Melanium* Ging. of the family Violaceae. According to Erben (1985), in the Southeast Europe, there are 50 species of Sect. *Melanium*, 28 of which are known to be located within the Republic of North Macedonia. The investigated species are autochthonous plants, endemic to

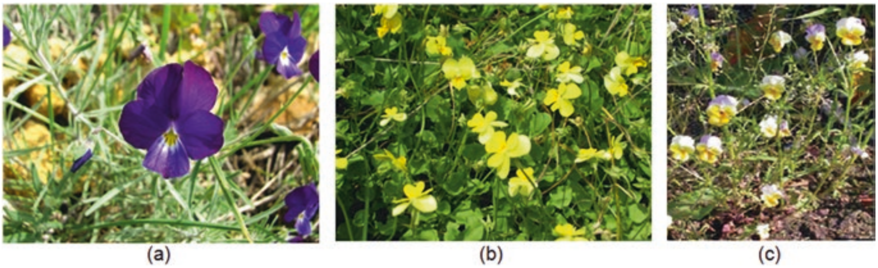


Fig. 13.2 *V. allchariensis* G. Beck (**a**), *V. arsenica* G. Beck (**b**), and *V. macedonica* Boiss. and Heldr. (**c**)

Fig. 13.3 *Thymus alsarensis* Ronniger

the Balkan Peninsula. *V. arsenica* and *V. allchariensis* grow only in the locality of Allchar and are considered to be local endemic species, while *V. macedonica* is widespread in central and southern regions of the Balkans and belongs in to the Balkan endemic species group (Micevski 1995).

The genus *Thymus* is one of the most polymorphic genera of the family Lamiaceae. Species of this genus are characterized by emphasized polymorphism and the presence of a number of subspecies, varieties, and forms (Fig. 13.3). *Thymus* flora is very rich and diverse in the territory of the Republic of North Macedonia (Matevski 1991). *T. alsarensis* Ronn. of the genus *Thymus* L., Sect. *Marginati* (A. Kerner) A. Kerner Subsect. *Verticillati* (Klok. et Shost.) Menitsky is an endemic aromatic and medicinal plant species present in the Allchar locality (Ronniger 1924). Its holotype is kept in the Herbarium of Natural History Museum in Vienna. Wild *thyme* species is used as an expectorant, antiseptic, anti-spasmolytic, and anthelmintic (Kulevanova et al. 1996). Infusion and decoction of aerial parts of *Thymus* species are used to produce tonics, digestive carminatives, expectorants, aids, antispasmodics, and anti-inflammatories. Furthermore, such preparations of

Fig. 13.4 *Centaurea leucomalla* Bornm. (**a**), *Onobrychis degenii* Dörfler (**b**), Knautia caroli-rechingeri Micev. (**c**), and Centaurea kavadarensis Micev. (**d**)

the medicinal plant species often are used for treatment of the common cold in Macedonian traditional medicine.

The species *Centaurea leucomalla* Bornm. grows at the same site, around the abandoned Allchar As-Sb-Tl mine. The species *Centaurea leucomalla* (fam. Compositae, section Phalolepis) is described by Bornmüller (1921) at the beginning of the twentieth century, one of the best experts on the flora of the southern parts of the Balkan Peninsula at that time. The investigated specimen is an autochthonous plant that grows only in the Allchar locality and is considered to be a local endemic species (Fig. 13.4). *Centaurea leucomalla* is considered as taxonomically a very stable plant species accepted in contemporary floristic literature such as Euro+Med Plantbase (https://ww2.bgbm.org/EuroPlusMed/query.asp).

Onobrychis degenii Dörfler (Fig. 13.4) is a significant plant species on the Red List of Threatened Plants of the IUCN (International Union for Conservation of Nature) (Walter and Gillett 1998), which is of indefinite status. This plant species grows only at the Allchar locality, near the village Majdan. However, it could be a Macedonian (sub)endemic species, though in floristic literature also is stated for Bulgaria (Kozhukharov 1976, 1992). *Knautia caroli-rechingeri* Micev. (Fig. 13.4) is a local Macedonian endemic plant species that is described by Micevski (1981), found only in the vicinity of the village Majdan, Allchar locality, and represents his *locus classicus*. This plant occupies a very isolated position in relation to other plant species of the *Knautia* genus that are developed on the territory of the Republic of North Macedonia, so that in the taxonomic relation it is closest to the *Knautia dinarica* species. For this time being, this species is known only for two sites located near the village of Majdan. *Centaurea kavadarensis* (Fig. 13.4) belongs to the *Centaurea deusta* group, and it is a local endemic plant species that can be found in the surroundings of the village Majdan, Allchar locality (Micevski 1987).

13.2.3 Soil and Plant Sampling and Sample Preparation

Plant and soil samples were gathered in the summer of 2011, at the Majdan locality around the abandoned mine Allchar, described above (Fig. 13.1). Soil samples were selected based on the presence of the endemic plant species. At each study site, soil samples (5–10 replicates from the soil represent composite material for one sample for analysis) were taken from the rhizosphere of plant specimens. *Viola* species collected from six sampling points, plant and soil samples were gathered at the locality around the abandoned mine described above. It must be mentioned that in the period when these plants samples were collected, only three samples had seeds. *Thymus* species and soil samples were collected at 14 locations in the investigated area, while the samples of *Centaurea leucomalla* species from eight sampling points. During the summer of 2011, *Onobrychis degenii, Knautia caroli-rechingeri*, and *Centaurea kavadarensis* species were collected from one sampling point. All of the gathered plant samples were washed carefully with redistilled water to remove soil particles and then separated into different parts (roots, stems, leaves, flowers, and seeds).

Collected soil samples were dried at room temperature for 5–7 days, and after extraneous material was removed, soil samples were sown using a plastic sieve with a 2 mm mesh. A certain amount of the sieved material was grinded into an agate mill until a powder, having a particle size of 0.125 mm, was obtained (Salminen et al. 2005). Such prepared samples were gathered in polyethylene bags ready for chemical analysis. Digestion of soil samples was performed by taking exact weighted soil sample (0.2500 g), placed in a PTFE digestion vessel and digested on a hot plate at about 100 °C. Digestion was performed in three steps. First, HNO3 was added to remove all organic matter. Then, a mixture of HF and HClO4 was added, and to the end, HCl and redistilled water were added to dissolve the residue. The digested solution was transferred quantitatively into 25 ml volumetric flask and then was analyzed.

Plant material was gathered according to the presence of the plant of the endemic species (10–20 specimens of plants represent composite material for one sample for analysis). After gathering, the plants were transferred to the laboratory. The whole plant was very carefully washed with redistilled water in order to remove the accumulated impurities from the ground and then split into the root, stem, leaf, and flower. The plant material after drying at room temperature for 7 to 14 days to a constant mass was pulverized, homogenized, and prepared for total digestion.

A specific mass (0.5000 g) of each plant sample was weighed into PTFE container to which 7 ml of concentrated HNO_3 and 2 ml H_2O_2 (30%, m/V) were added; then the PTFE vessels were placed in a microwave digestion system (CEM, Mars). In the first step of the previously established method, the temperature of 180 °C was reached for about 15 min and then heated at the same temperature for an additional 20 min. Then, the PTFE vessels were removed from the microwave digestion system when the sample temperature was close to room temperature. The solutions obtained after the thermal decomposition of the plant tissues were quantitatively transferred into 25 ml calibrated flasks.

To determine the degree of extraction (bioavailability) of the analyzed soil elements, three different extraction solutions were used (Pansu and Gautheyrou 2006):

- Extraction with water. With this extraction, the natural conditions for extraction of toxic elements from the soil were simulated, to determine which elements can be extracted from the soil if it rains in nature under normal conditions.

- Reagent for extraction of diethylenetriaminepentaacetic acid-calcium chloride-triethanolamine (DTPA-$CaCl_2$-TEA). This extraction solution is very often used to determine the extraction of trace elements or biogenic elements which the plant most often uses for its growth and development.
- Dissolution with low pH value was used to determine potentially accessible forms that were difficult to extract from the soil. In this case, 0.1 M HCl solution was used.

With the simulation of these extraction solutions, we managed to determine the level of available soil elements where plants were being developed.

13.2.4 Instrumentation

All analyzed elements were determined by the application of atomic emission spectrometry with inductively coupled plasma, ICP-AES (Varian, 715- ES), applying additional ultrasonic nebulizer CETAC (ICP/U-5000AT$^+$) for better sensitivity. For this investigation, certified reference materials were used to validate the method for all considered elements, and the difference between measured and certified values was satisfied ranging within 15%. Thus, the quality control was ensured by standard moss reference materials M2 and M3 (Steinnes et al. 1997) as well as JSAC 0401 soil reference material. The measured concentrations were in good agreement with the recommended values.

13.3 Results and Discussion

13.3.1 Viola Species

The content of all analyzed elements (Ag, Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sb, Sr, Tl, V, and Zn) of all three endemic *Viola* species (*V. allchariensis, V. arsenica*, and *V. macedonica*) were obtained (Bačeva et al. 2014a). For the comparison in Table 13.1, data for the median content of As, Sb, and Tl and their minima and maxima in different plant parts (roots, stems, leaves, flowers, and seeds) are presented.

Because all three *Viola* species grow at the Allchar locality and this endemic plant species are exposed to excess amounts of As, Sb, and Tl that are present in the soil (Volkov et al. 2006; Boev and Jelenkovic 2012; Bačeva et al. 2014b), particular attention was given to the content of As, Sb, and Tl. Data about the content of these elements in different parts of the *Viola* species collected from all six locations are given in Table 13.1. Mass fractions of As, Sb, and Tl in different plant parts of *Viola* species collected from all six locations are presented in Fig. 13.5a–c.

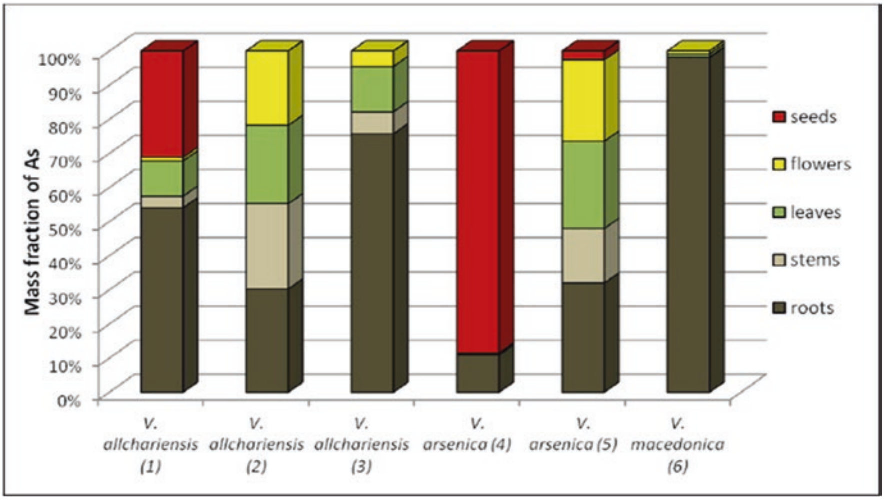
Table 13.1 The content of As, Sb, and Tl in different parts of the *V. allchariensis*

Element	Root		Stem		Leaf		Flower		Seed	
Viola allchariensis										
	Med	Min-max	Med	Min-max	Med	Min-max	Med	Min-max	Med	Min-max
As	24.3	4.1–373	3.40	2.08–23.1	4.26	3.12–71.5	2.96	1.45–7.19	108	1.45–215
Sb	0.55	0.25–0.57	0.25	0.25–0.52	0.50	0.25–0.63	0.25	0.25–0.25	0.40	0.25–0.55
Tl	311	15–1215	459	9.72–842	2192	18.9–4013	975	36–2907	1690	472–2907
Viola arsenica										
As	211	77.8–343	21.9	4.82–38.9	32.3	2.41–62.3	34.3	10.6–58.0	1392	6.3–2776
Sb	1.06	0.98–1.14	0.25	0.25–0.25	0.72	0.50–0.93	0.47	0.25–0.69	0.91	0.25–1.57
Tl	1877	1653–2102	2831	2649–3013	9092	7780–10,404	2669	2566–2771	1877	1388–2367
Viola macedonica[a]										
As	158		0.25		1.46		1.38			
Sb	0.25		0.25		0.25		0.25			
Tl	356		567		4292		2310			

Values are given in mg kg^{-1}

Med median, *Min* minimum, *Max* maximum

[a] One location



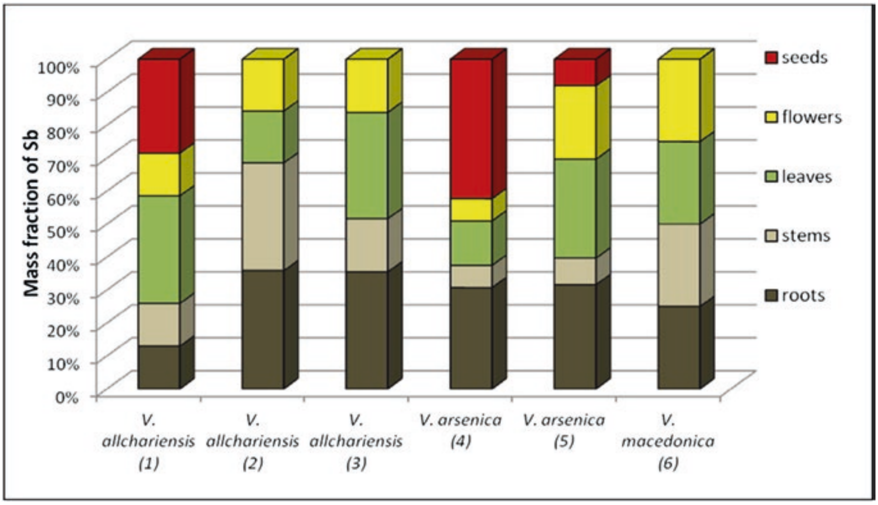


Fig. 13.5 (**a**) Mass fraction of As, Sb, and Tl in different plant parts of *Viola* species – arsenic. (**b**) Mass fraction of As, Sb, and Tl in different plant parts of *Viola* species – antimony. (**c**) Mass fraction of As, Sb, and Tl in different plant parts of *Viola* species – thallium

The highest accumulation of As was found in seeds of *V. arsenica* with a median value of 1392 mg kg^{-1} (mass fraction of 2.5–88.5%) and in the roots of *V. arsenica* with the median value of 211 mg kg^{-1} (mass fraction of 10–31%). The median value was 158 mg kg^{-1} (mass fraction of 98%) for the roots of *V. macedonica*, and the lowest median value was in the roots of *V. allchariensis* with 24.3 mg kg^{-1} (mass fraction of 30–75.7%). For their aboveground parts, the accumulation of As in the stems was found to be highest in the *V. arsenica* with a median value of 21.9 mg kg^{-1} (mass fraction to 16%), followed by *V. allchariensis*, with median of 3.40 mg kg^{-1} (mass

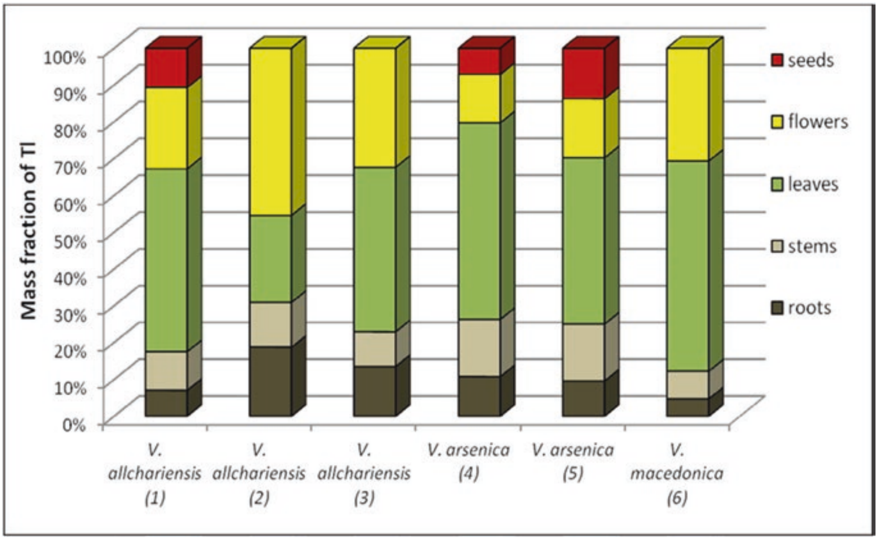


Fig. .13.5 (continued)

fraction to 25%), and lowest in the stems of *V. macedonica* with 0.25 mg kg^{-1} (mass fraction of 0.16%). The accumulation of As in the leaves and flowers in all three plant species was low (<40 mg kg^{-1}), but the accumulation of As was higher in the seeds; in *V. arsenica* the values were in ranges of 6.34 to 2776 mg kg^{-1} (median of 1392 mg kg^{-1}) and the lowest in *V. allchariensis* with a median value of 108 mg kg^{-1} (mass fraction of 31.2%). There were no seeds available for *V. macedonica* during the sample collection period. Despite growing at spots with higher available As, the content of this toxic element was considerably higher in the roots and seeds.

From data given in Table 13.1, it can be noticed that the content of Sb in all plant parts was low and it had no significant differences between the different parts of the plant. For the accumulation of Sb in all three *Viola* species, we can observe that the majority of accumulated Sb was in the roots, seeds, and leaves of the *V. arsenica* with the median value of 1.06, 0.91, and 0.72 mg kg^{-1} (mass fraction 30, 42, and 30%), respectively. The accumulation of Sb in the *V. allchariensis* and *V. macedonica* was very low (<0.60 mg kg^{-1}).

In this study one of the main objectives was on the accumulation of Tl in relation to its mobility in *Viola* species. It was noticed that the content of Tl was significantly higher in the leaves and flowers (Table 13.1, Fig. 13.5a–c). The highest Tl accumulation was found in leaves, flowers, and seeds in *V. arsenica* with median values of 9092 mg kg^{-1}; 2669 mg kg^{-1}, and 1877 mg kg^{-1} (mass fractions of 45–53%; 13–16%, and 13.7%), respectively. For *V. allchariensis* the median values were 2192 mg kg^{-1}; 975 mg kg^{-1}, and 1690 mg kg^{-1} (mass fractions are 23.7–49.7%; 22–45.4%, and 10%), respectively. For *V. macedonica* the median values of Tl in leaves and flowers were 4292 mg kg^{-1} and 2310 mg kg^{-1} (mass fractions are 57% and 30.7%), respectively. The content of Tl in the roots and stems in all *Viola* plant species was also very high. Thus, its median content in the roots and stems of

V. arsenica was 1877 mg kg^{-1} and 2831 mg kg^{-1}, for *V. allchariensis* 312 mg kg^{-1} and 459 mg kg^{-1}, and for *V. macedonica* 365 mg kg^{-1} and 567 mg kg^{-1}, respectively. The appropriate mass fractions of Tl in roots and stems of *V. arsenica* were 10% and 15.5%, for *V. allchariensis* 7–18.7% and 9.4–12.2%, and for *V. macedonica* 4.7% and 7.5%, respectively.

For animals and humans, the major pathway of exposure is the ingestion of plant species that has grown on Tl-contaminated soils (Scheckel et al. 2004). When Tl is present in soils, it may be easily taken up by plants because it is generally present as thermodynamically stable Tl(I), an analogue of potassium (Madejon et al. 2007). Thallium(I) has a very low stability that is constant with both organic and inorganic ligands (Kaplan and Mattigod 1998; Nriagu 1998) and therefore should be easily extractable from the leaves (Scheckel et al. 2004). However, transport of K and Tl appeared to occur in different ways because Tremel et al. (1997) cannot find any correlation between Tl and K in seeds and suggested that the explanation of the behavior of Tl in the current study is probably similar to these indications.

From the results given in Table 13.1 and Fig. 13.5a–c, it can be seen that all of the *Viola* species are able to accumulate toxic elements and distribute them into all plant parts, especially with regard to the accumulation of As and Tl. The content of As was significantly higher in the roots and seeds, but the content of Sb was considerably lower compared with the content of As and Tl. With no significant differences between the different parts of the plant, the content of Tl was significantly higher in the leaves and flowers in all *Viola* species.

For the *Viola* endemic species, previous investigations were done, but only for the accumulated As (not for Sb and Tl), and it was pointed out that the content of As was significantly higher in the compared roots (Stevanović et al. 2010). It should be emphasized that in the work of Stevanović et al. (2010) the plant species are divided in roots and shoots and analyzed only for As.

All soil samples were collected from the same locations as the *Viola* species, and they were also analyzed for the same elements (Table 13.2). It can be seen that all three *Viola* species were exposed to the excess amount of As present in the soil, and the accumulation of As in this endemic species is significantly high for that reason.

From the results given in Table 13.2, it can be noticed that the content of As in soil samples was in the range from 86 to 12,775 mg kg^{-1} with the median value of 5875 mg kg^{-1}. The content of the Sb in the same soil samples ranged from 21 to 116 mg kg^{-1} (median 37 mg kg^{-1}), while the content of Tl ranged from 14 to 2140 mg kg^{-1} with the median of 431 mg kg^{-1}. Based on previous geological surveys of this locality, it may be concluded that such significantly high contents of these elements are not related to any anthropogenic activities and that their sources are mainly from natural phenomena (Volkov et al. 2006; Boev and Jelenkovic 2012). Namely, it is known that the presence of Tl in the ore from this region (the northern part of the locality of Allchar) ranges from 0.1 to 0.5%, with Sb up to 2.5% and As level of about 1.5% (Volkov et al. 2006).

Different degrees of availability can be estimated depending on the extracting power of the reagent that is used. We decided to investigate bioavailability of the analyzed elements by the soil *extraction with water,* which only provides informa-

Table 13.2 The content of As, Sb, and Tl in soil samples from the locations where *Viola* species were collected (in mg kg^{-1})

Element	Average	Median	Min.	Max.
As	5464	5875	86	12,775
Sb	47	37	21	116
Tl	595	431	14	2140

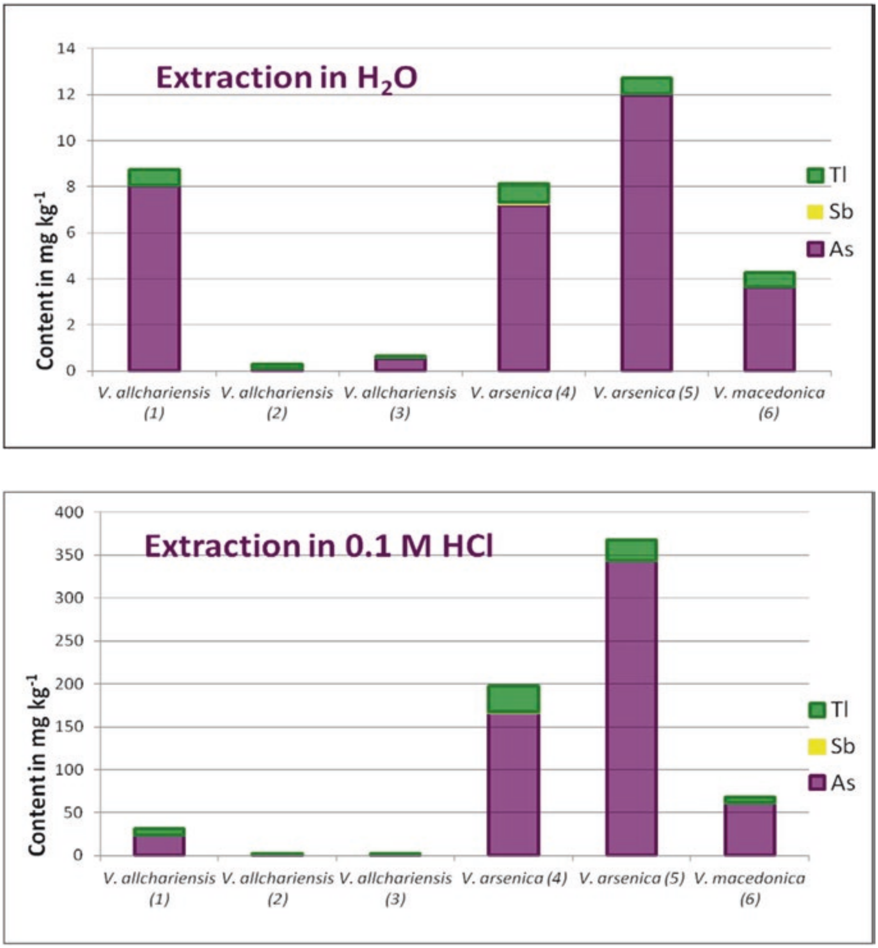


Fig. 13.6 (**a**) Extraction for plant-available As, Sb, and Tl in H_2O. (**b**) Extraction for plant-available As, Sb, and Tl in 0.1 M HCl. (**c**) Extraction for plant-available As, Sb, and Tl in DTPA-TEA-$CaCl_2$ extraction solutions

tion on the actual availability of elements from the soil solution (Pansu and Gautheyrou 2006). Also, the extraction that use *sequestering reagents* is widely used for selective solubilization by chelate formation, often in combination with other reagents acting by ion exchange, redox, or acid action. The most widely used sequestering reagents for the estimation of the extraction of trace elements include

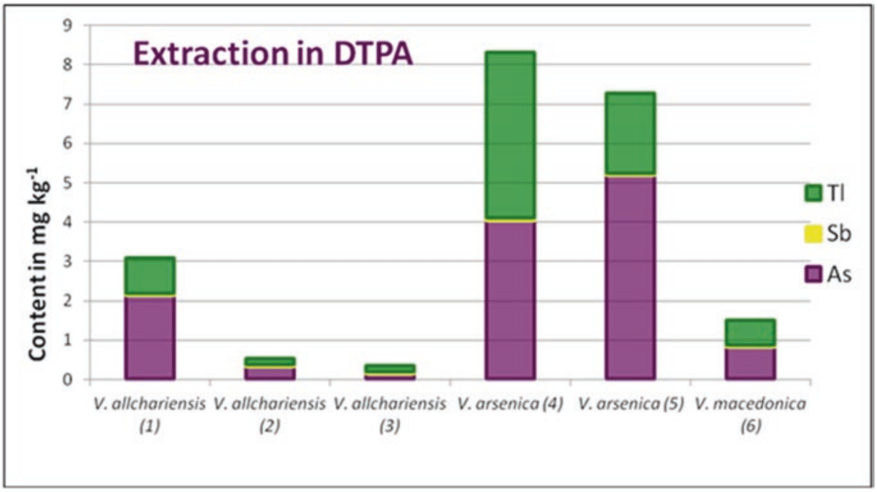


Fig. .13.6 (continued)

DTPA action (DTPA-CaCl2-TEA), which is often recommended for the extraction of toxic or biogenic metals (Risser and Baker 1990; Pansu and Gautheyrou 2006). Additionally, *acid reagents* are often used to displace potentially available forms that are not easily extracted (Risser and Baker 1990; Pansu and Gautheyrou 2006). In our case a solution of 0.1 M HCl was applied. With the simulations of these extractions, we were able to assess the level of plant-available elements and conditions in live plants.

The level of extraction or the level of plant-available As, Sb, and Tl from the soil samples collected from locations where *Viola* species were taken was given in Fig. 13.6a–c. It can be seen that As was the most readily available from the soil samples in the HCl extraction solution. Thus, in soil from the locations of *V. arsenica*, the amount of 344 mg kg^{-1} of As was extracted which represented about 2.5% of the As present in the soil. Slightly lower levels of As were extracted from the soil where the endemic plant *V. macedonica* was gathered (61.5 mg kg^{-1} or 1.25% extractability), while the less extractible As was from the soil from the *V. allchariensis* location (0.20–1.0% extractability). The extractability of Tl was very similar for soils where all *Viola* species were gathered (about 2%); only for *V. arsenica* locations, varying amounts of Tl from 0.6 mg kg^{-1} to 30.3 mg kg^{-1} were extracted. The extractability of Sb from the soil samples in the extraction solution of 0.1 M HCl was lower than those for As and ranged from 0.2% for soil from *V. allchariensis* locations to 0.82% for the soil from *V. arsenica* locations.

Table 13.3 The content of As, Sb, and Tl in *Thymus alsarensis* (in mg kg^{-1})

	Thymus alsarensis Ronn.			Soil (0–5 cm)		
Element	Average	Med	Min-max	Average	Med	Min-max
As	16.1	3	0.25–140	809	215	35–5290
Sb	0.4	0.25	0.25–1.51	34.7	23	6.3–130
Tl	95.4	20.7	0.10–496	58.8	15.3	2.0–330

Table 13.4 Average values for As, Sb, and Tl in different parts of the *Thymus alsarensis* (in mg kg^{-1})

Elements	Root	Stem	Leaf	Flower
As	62.7	10.6	14	4.45
Sb	0.89	0.29	0.43	0.3
Tl	147	60.7	104	131

*13.3.2 Thymus Species (*Thymus alsarensis *Ronniger)*

Because the endemic plant species *thyme* grows at the Allchar deposit and is exposed to excess amounts of As, Sb, and Tl that are present in the soil (Volkov et al. 2006; Boev and Jelenkovic 2012; Bačeva et al. 2014b), particular attention was given also to the content of these elements in plant samples. The content of some other heavy metals in *Thymus alsarensis* Ronniger was previously determined in the whole plant (Kadifkova Panovska et al. 1996; Bačeva et al. 2016). As the most present toxic elements in the soil of the Allchar locality (Bačeva et al. 2014b), here data about the content of As, Sb, and Tl in *T. alsarensis* and in the surrounding soil are presented in Tables 13.3 and 13.4.

The median value of As, Sb, and Tl from various sampling sites was 215, 23, and 15 mg kg^{-1}, respectively. Total content of As in soil samples from various sites where *Thymus* species were collected varied remarkably from 34.7 to 5290 mg kg^{-1}, with a median of 215 mg kg^{-1}. Total Sb content in the soil where samples of *T. alsarensis* were taken varied from 6.3 to 130 mg kg^{-1}, with a median value of 23 mg kg^{-1} (Table 13.3). The total content of Tl in soil where samples of *T. alsarensis* were taken varied from 2.0 to 330 mg kg^{-1}, with a median of 15.3 mg kg^{-1}.

From the results that are presented in Tables 13.3 and 13.4, it can be seen that the content of Tl and As in some plant samples that are collected in this area are significantly elevated (up to 140 mg kg^{-1} and 496 mg kg^{-1} for As and Tl, respectively). Based on Tables 13.3 and 13.4, as well as Fig. 13.7, it is evident that samples of *T. alsarensis* were able to accumulate toxic elements, especially As and Tl, and distribute them in all parts of the plant. The content of As was significantly higher in roots and leaves, but the content of Sb was considerably lower than both of As and Tl, and it has no significant variation between the content in different parts of the plant. The content of Tl was significantly higher in the roots, flowers, and leaves of all samples of *T. alsarensis* that were collected from the Allchar locality.

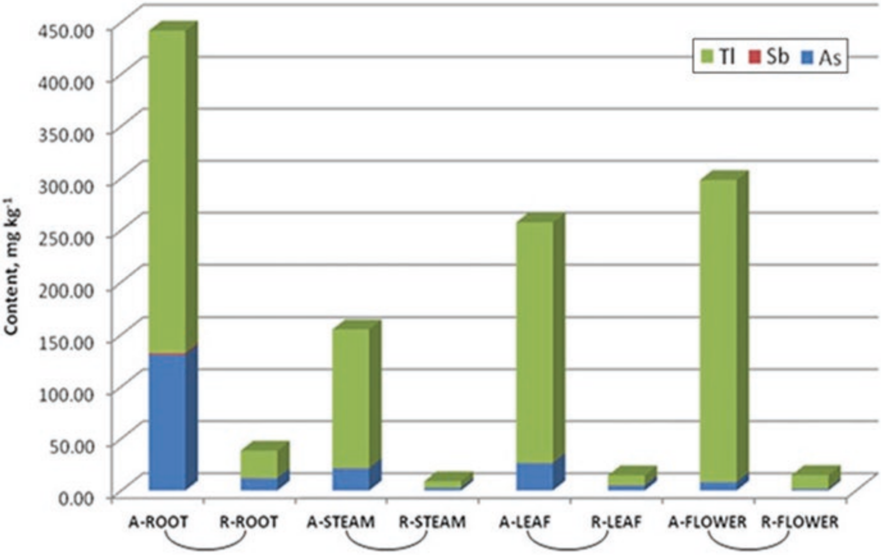


Fig. 13.7 Comparison between samples of *Thymus alsarensis* collected from the Allchar area (A area around the Allchar mine, R rest of the Allchar area)

All endemic *T. alsarensis* species that grow in the vicinity of the Allchar locality appear to be exposed to excess amounts of As that is present in the soil (Table 13.3). As such, significant accumulation of As in these endemic species was observed. The highest accumulation of As in *T. alsarensis* was found in the roots, with an average value of 62.7 mg kg^{-1} (Table 13.4). For the aboveground parts of these endemic plant species, the highest average value of 14 mg kg^{-1} was found in the leaves of the plant, while the average value for the stems was 10.6 mg kg^{-1}. The lowest content of As was observed to be in the flowers.

All samples of *T. alsarensis* that were collected and identified in this area were exposed to soil containing high amounts of Sb as well. From Table 13.4, it is apparent that the content of Sb in all plant parts was low. Furthermore, no significant differences between the different parts of the plant were observed. The majority of Sb accumulated in the roots and leaves, and the average values for these parts were 0.89 mg kg^{-1} and 0.43 mg kg^{-1}, respectively. Accumulation of Sb in the flowers and stems was minimal <0.30 mg kg^{-1}. The comparison of the content of Tl in different parts of the plant indicated that significantly higher content of Tl was observed in the roots and flowers (Table 13.4). Accumulation of Tl in roots, flowers, and leaves in *T. alsarensis* had an average value of 147, 131, and 104 mg kg^{-1}, respectively. In stems the average value was 60.7 mg kg^{-1}.

Comparison of samples that were collected near or around the Allchar mine ($n = 5$) with samples from the rest of the area ($n = 9$) revealed significant differences in the content of As and Tl (Fig. 13.7). Thus, the average content of As in the roots (A-Root) of samples around the Allchar mine was 139 mg kg^{-1}, which is nearly seven times higher than values of the samples from the rest of the area (average value of 20.1 mg kg^{-1}). Differences between the content of As in stems and leaves from samples around the Allchar mine (19.7 mg kg^{-1} and 22.7 mg kg^{-1}, respectively) compared with samples from the rest of the area (5.61 mg kg^{-1} in stems and 9.15 mg kg^{-1} in leaves) were also evident.

The content of Tl was significantly higher compared with As and Sb in all parts of the plant. Accumulation of Tl was much higher in the roots collected from the (A-R) area, with the average content being 317 mg kg^{-1}, compared with roots from the (R-R) area, where the average value was 53.6 mg kg^{-1}. Remarkable differences between the contents of Tl in aboveground parts of the plant (flowers, leaves, and stems) were evident from samples collected around the Allchar mine (262, 172, and 89.7 mg kg^{-1}, respectively) compared with samples from the rest of the area (58.8, 66.5, and 44.5 mg kg^{-1}, respectively). Antimony had considerably less accumulation compared with As and Tl, and it had no significant variation between the different parts of *T. alsarensis* collected from the Allchar locality (Fig. 13.7). Less accumulation and lower content of Sb in samples of *T. alsarensis* were due to fact that the Sb mineralization (mainly mineral stibnite) was distributed in deeper layers in the mine deposit (Jelenković and Boev 2011).

The extraction tests of soil show that Tl was extracted with extraction solutions of HCl of about 1.2% of the Tl present in the soil (Fig. 13.8). Lower levels of As were extracted from soil (0.84%), while Sb was the least extractible (0.81% extractability). The extractability of As, Sb, and Tl in an extraction solution of H_2O (pH = 7) and DTPA-$CaCl_2$-TEA was low (below 0.2% for all elements).

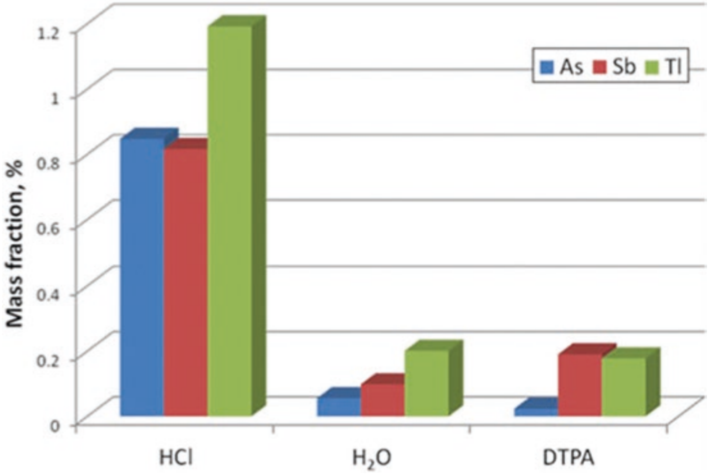


Fig. 13.8 Plant-available As, Sb, and Tl in 0.1 M HCl, H_2O, and DTPA+TEA+$CaCl_2$ extraction solutions

13.3.3 Centaurea Species

13.3.3.1 *Centaurea leucomalla* Bornm.

The content of 22 elements (As, Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, Sb, Sr, Tl, V, and Zn) in the endemic *Centaurea leucomalla* Bornm. species collected from 8 sublocations is presented by Bačeva et al. (2018a) in Table 13.5. The average, median, minimum, and maximum values of the content of As, Sb, and Tl in soil and plant samples are presented in Table 13.5, while their content in different plant parts (root, stem, and leaf) of *Centaurea leucomalla* is given in Table 13.6. It can be seen that the average content of As in soil samples was 1801 mg kg^{-1} and ranges from 27 to 5288 mg kg^{-1} (Table 13.5). The average content of Sb in soil samples, from the same locations where the samples of *Centaurea leucomalla* were collected, was 2079 mg kg^{-1} and ranges from 8.66 to 8816 mg kg^{-1} (Table 13.5). From the data of the soil samples, it was establish that the average content of Tl was 271 mg kg^{-1}, and it ranges from 22.2 to 530 mg kg^{-1}.

From the obtained results (Tables 13.5 and 13.6), it can be seen that the content of Tl and As in the plant samples was significantly high (up to 210 mg kg^{-1} for As and up to 238 mg kg^{-1} for Tl). These results confirm that the *Centaurea leucomalla* was able to accumulate toxic elements, especially As and Tl, and distribute them in all plant parts. The content of As was significantly higher in the roots and leaves (Table 13.6). However, even though the content of Sb in the soil was high (Table 13.6), its content in the plant samples was low compared with As and Tl, and it has no significant variation between the different parts of the plant.

The results of the extraction levels of As, Sb, and Tl from the soil samples gathered from locations where *Centaurea* samples were taken are presented in Fig. 13.9. It could be seen that the highest extractability of these elements was with the extraction solution of 0.1 M HCl, with the extractable content of 6.27 mg kg^{-1}, which was 2.3% of total content of Tl in the soil. The extraction levels of As was 0.36% (6.5 mg kg^{-1}), while the extractable level of Sb was the lowest (6.08 mg kg^{-1} or 0.29%). The extractability of As and Sb in water (pH 7) and DTPA-$CaCl_2$-TEA was very low (less than 0.1% for As and Sb), while the extraction of Tl in these extraction solvents was slightly higher than other elements (0.32 and 0.84%, respectively).

The biotransfer of this element in the *Centaurea leucomalla* was very high with the average content in the plant of 59.5 mg kg^{-1} ranging from 0.55 to 210 mg kg^{-1}. The highest content was found in the roots (average value of 92.3 mg kg^{-1}, ranges

Table 13.5 The results from the analysis of As, Sb, and Tl elements in plant *Centaurea leucomalla* Bornm. and corresponding soil (in mg kg^{-1})

	Centaurea leucomalla Bornm.			Soil (0–5 cm)		
Elements	Average	Median	Min-max	Average	Median	Min-max
As	59.48	28.4	0.55–210	1801	1655	26.9–5288
Sb	6.94	0.74	0.25–58.2	2079	65.6	8.66–8816
Tl	42.1	20.9	0.10–238	271	289	22.2–530

Table 13.6 Data of the content of As, Sb, and Tl in various parts of the plant *Centaurea leucomalla* Bornm. (in mg kg^{-1})

	Root			Stem			Leaf		
Elements	Average	Median	Min-max	Average	Median	Min-max	Average	Median	Min-max
As	92.3	103	2.39–204	22.1	8.85	0.55–91.5	63.9	20.6	1.59–210
Sb	7.57	1.14	0.25–54.4	5.52	0.74	0.25–37.7	7.74	0.38	0.25–58.2
Tl	86.3	63.1	10.3–238	17.4	9.93	1.86–69.1	22.5	13.8	0.10–75.2

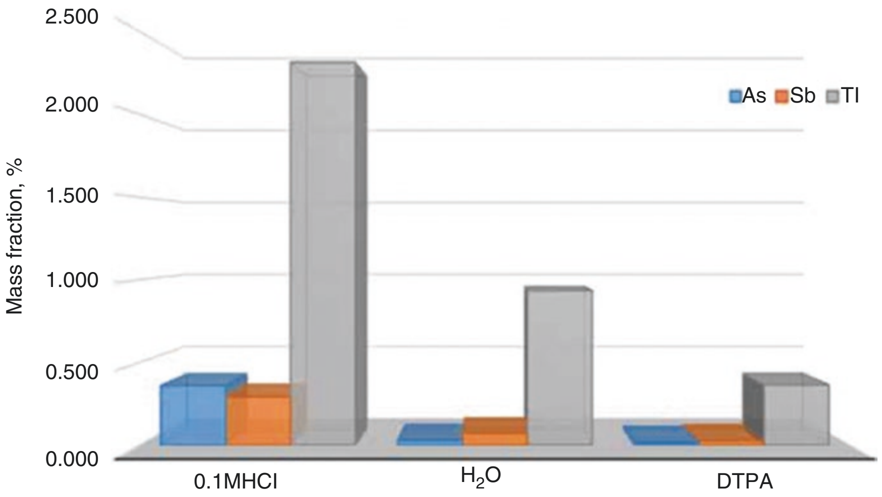


Fig. 13.9 Plant-available As, Sb, and Tl in 0.1 M HCl, H_2O, and DTPA+TEA+$CaCl_2$ extraction solutions of soil from locations where *Centaurea* samples were taken

from 2.39 to 204 mg kg^{-1}), then in the leaves (average value of 63.9 mg kg^{-1}, ranges from 1.59 to 210 mg kg^{-1}) with the lowest average content in the stem (22.1 mg kg^{-1}, ranges from 0.55 to 91.5 mg kg^{-1}). However, despite the high content of Sb in the soil, the content of Sb in the plant was low (average content of 6.94 mg kg^{-1}, ranges from 0.25 to 58.2 mg kg^{-1}), compared with the values for As and Tl in the same plants.

From the plant material that was analyzed, despite the As and Sb, special attention was given to the accumulation and transfer of Tl in different parts of the species *Centaurea leucomalla*, and high bioaccumulation by the plants was found (average content in the plant of 42.1 mg kg^{-1}, ranges from 0.1 to 238 mg kg^{-1}). The accumulation of Tl follows the behavior of As with its high content in all parts of the plant, the highest in the root (86.3 mg kg^{-1}, ranges from 10.3 to 238 mg kg^{-1}) than in the leaf (average of 22.5 mg kg^{-1}, ranges from 0.1 to 75.2 mg kg^{-1}) and the lowest in the stem (average content of 17.4 mg kg^{-1}, ranges from 1.86 to 69.1 mg kg^{-1}).

13.3.3.2 *Centaurea kavadarensis* Micev.

From the results presented in Table 13.7, it can be noticed that the contents of As, Sb, and Tl in the soil sample gathered from the site where *Centaurea kavadarensis* Micev. samples were collected were much lower than in the soil samples from the sites of the other two plant species (Bačeva et al. 2014a), and therefore the accumulation of these elements was much smaller in this plant than in the other species from Allchar locality. Probably the reason for that is that the plant samples of the other endemic species were taken in the area close to the mine or a hydrothermal-modified rock area, where the mineralization of As and Tl was predominant, and

Table 13.7 The content of As, Sb, and Tl elements in different parts if plant *Centaurea kavadarensis* and corresponding soil (in mg kg^{-1})

Element	Soil	Root	Stem	Leaf	Flower
As	102	<0.50	<0.50	<0.50	<0.50
Sb	8.40	<0.50	<0.50	<0.50	<0.50
Tl	4.45	4.31	4.13	5.38	3.18

Table 13.8 The content of As, Sb, and Tl elements in different parts of plants *Onobrychis degenii* and *Knautia caroli-rechingeri* and corresponding soil (in mg kg^{-1})

	Onobrychis degenii					*Knautia caroli-rechingeri*			
Element	Soil	Root	Stem	Leaf	Flower	Soil	Root	Stem	Leaf
As	5288	114	0.58	4.03	3.49	4932	354	1.66	3.78
Sb	80.7	<0.50	<0.50	<0.50	<0.50	30.9	0.68	<0.50	<0.50
Tl	330	86.7	6.34	6.83	6.19	409	216	26.3	10.4

opposite of them, the plant samples of *Centaurea kavadarensis* was taken 5.5 km from the hydrothermal-altered stones along the river of Majdanska Reka.

13.3.4 Onobrychis degenii *Dörfler and* Knautia caroli-rechingeri *Micev.*

After statistical process of the analyzed results for plant specimens of the endemic plant species *Onobrychis degenii* Dörfler and *Knautia caroli-rechingeri* Micev. and the corresponding soil samples, the data of the 27 analyzed elements from different parts of the plant (root, stem, leaf, and flower) are obtained (Bačeva et al. 2018b). In Table 13.8 data for the content of As, Sb, and Tl are presented. Plant species *Onobrychis degenii* and *Knautia caroli-rechingeri* were taken in the Crven Dol area or a hydrothermal-modified rock area, where the mineralization of As and Tl was predominant.

The content of As in the root of *O. degenii* was 114 mg kg^{-1} and for *K. caroli-rechingeri* was 354 mg kg^{-1}. The content of As in the aboveground parts of the plant *O. degenii* was 4.03 mg kg^{-1} in the leaf and 3.49 mg kg^{-1} in the flower. For *K. caroli-rechingeri*, the content of As in the leaf was 3.78 mg kg^{-1} (Table 13.8). The content of Tl in the root of these plants was also high, ranging from 86.7 mg kg^{-1} in the roots of *O. degenii* to 216 mg kg^{-1} in the roots of *K. caroli-rechingeri* (Table 13.8). For the other parts of the plants (stem, leaf, and flower), the content of Tl in *O. degenii* was 6.3 mg kg^{-1}, 6.8 mg kg^{-1}, and 6.19 mg kg^{-1}, respectively, while for the *K. caroli-rechingeri* the content of Tl in the stem, leaf, and flower was higher: 26.3 mg kg^{-1} and 10.4 mg kg^{-1}, respectively (Table 13.8). The content of Sb was significantly lower in different parts of the plants compared to the content of As and Tl (Table 13.8). The reason for this is due to the low content of Sb in the part of Crven

Dol locality, and therefore its accumulation by the plants was much lower. Extraction tests of As, Sb, and Tl from the soil samples show that the highest level of As was extracted with an extraction solution of 0.1 M HCl with 7.0% of As, 1.2% of Sb, and 1.2% of Tl.

13.3.5 Assimilation by Plants

Bioaccumulation is extremely important in the scientific evaluation of risks that toxic elements may pose to humans and the environment and is a current focus of regulatory effort. Using the results of the analysis of As, Sb, and Tl in different plant parts (roots, stems, leaves, flowers, and seeds) of the investigated endemic species, the biological accumulation factor (BAF), defined as the ratio between total content in certain plant (sum of the content in roots, stems, leaves, flowers, and seeds) and the content of the element in corresponding soils, and the biological transfer factor (BTF), defined as the ratio between the content of the element in flowers and that of the roots, were calculated in all endemic plant species that were gathered from the Allchar locality.

The average biological accumulation factor (BAF) for *Viola* (Table 13.9) for As was 0.17 and was higher than the previous data obtained by Stevanović et al. (2010). The average translocation factors (the ratio of the As concentration in the flowers, or seed, to the As concentration in the roots) were significantly high for *V. arsenica* and *V. allchariensis*. The BAF and BTF for Sb are insignificant, because its extractability from soil is negligible. The high BAF value of 33.8 as well as BTF value of 3.29 for Tl shows that *V. allchariensis* is capable to accumulate high amounts of Tl.

Assessment of the distribution and accumulation of As, Sb, and Tl in endemic *Thymus* plants is of great importance for environmental monitoring. Moreover, such plants are commonly used for medicinal and culinary purposes. Based on the results

Table 13.9 The mean content of As, Sb, and Tl in plant parts of *Viola* species and calculated bioaccumulation (BAF) and biotransfer (BTF) factors (in mg kg^{-1})

Sample	Root	Stem	Leaves	Flowers	Seeds	Sum	Soil	BAF	BTF
As									
V. allchariensis	134	9.54	26.3	3.87	108	282	2573	0.11	0.81
V. arsenica	210	21.9	32.3	34.3	1391	1691	10,066	0.17	6.61
V. macedonica	158	0.25	1.46	1.38	–	161	4932	0.03	0.01
Sb									
V. allchariensis	0.46	0.34	0.46	0.25	0.40	1.91	33.8	0.06	0.87
V. arsenica	1.06	0.25	0.72	0.47	0.91	3.40	70.8	0.05	0.86
V. macedonica	0.25	0.25	0.25	0.25	–	1.00	30.9	0.03	1.00
Tl									
V. allchariensis	514	437	2075	1306	1690	6022	178	33.80	3.29
V. arsenica	1877	2831	9092	2669	1877	18,346	1312	13.98	1.00
V. macedonica	356	567	4292	2310	–	7525	409	18.40	6.49

Table 13.10 The content of As, Sb, and Tl in different parts of *T. alsarensis* and the calculated BAF and BTF

Element	Root	Stem	Leaves	Flowers	Sum	Soil	BAF	BTF
As	62.7	10.6	14.0	4.45	91.8	809	0.114	0.071
Sb	0.89	0.29	0.43	0.30	1.90	34.7	0.055	0.336
Tl	147	60.7	104	131	444	58.8	7.556	0.891

Table 13.11 The content of As, Sb, and Tl and the values of BAF and BTF for the different parts of *Centaurea leucomalla* (in mg kg^{-1})

Element	Root	Stem	Leaf	Sum	Soil	BAF	BTF
As	92.3	22.1	63.9	178	1801	0.099	0.693
Sb	7.57	5.52	7.74	20.8	2079	0.010	1.022
Tl	86.3	17.4	22.5	126	271	0.466	0.261

Table 13.12 Results from the analysis of different parts of *Onobrychis degenii*, *Knautia caroli-rechingeri*, and Centaurea kavadarensis and bioaccumulation and biotransfer factors (in mg kg^{-1})

Species	Root	Stem	Leaf	Flower	Sum	Soil	BAF	BTF
As								
Onobrychis degenii	114	0.58	4.03	3.49	122	5288	0.023	0.031
Knautia caroli-rechingeri	354	1.66	3.78	–	360	4932	0.073	0.011
Centaurea kavadarensis	<0.50	<0.50	<0.50	<0.50	–	102	–	–
Sb								
Onobrychis degenii	<0.50	<0.50	<0.50	<0.50	–	80.7	–	–
Knautia caroli-rechingeri	0.68	<0.50	<0.50	–	0.68	30.9	0.022	–
Centaurea kavadarensis	<0.50	<0.50	<0.50	<0.50	–	8.40	–	–
Tl								
Onobrychis degenii	86.7	6.34	6.83	6.19	106	330	0.322	0.071
Knautia caroli-rechingeri	216	26.3	10.4	–	253	409	0.618	0.048
Centaurea kavadarensis	4.31	4.13	5.38	3.18	17.0	4.45	3.819	0.737

of the analysis of As, Sb, and Tl in different plant parts (roots, stems, leaves, and flowers) of *Thymus* endemic species that grow in the Allchar mining area, BAF and BTF were calculated (Table 13.10).

For As and Sb in this endemic species, BAF was insignificant. For Tl, BAF was 7.5, which was significantly higher. BTF for Tl in samples of *Thymus* was 0.89, which was smaller than the BAF. Data in Table 13.10 show that BAF for the Tl was significantly higher than that for As, showing that *T. alsarensis* was capable to accumulate high amounts of Tl than As and Sb.

The calculated values for BAF and BTF for the contents of As, Sb, and Tl in various parts of the plant (root, stem, and leaf) of *Centaurea leucomalla* are presented in Table 13.11. It was obvious that the BAF values for As and Sb were insignificant, while this factor for Tl was the highest (BAF = 0.46). This indicates that the endemic *Centaurea leucomalla* from Allchar accumulates thallium much more intensively than arsenic and antimony. Unlike BAF, the highest BTF in *Centaurea leucomalla* for Sb has a value of 1.02. From the other side, BTF was higher for As and Sb showing that the transfer of these elements from roots to the flower was more significant than Tl.

Table 13.12 presents the results of the contents of As, Sb, and Tl in soil and in all parts of *Centaurea kavadarensis*, *Onobrychis degenii*, and *Knautia caroli-rechingeri* and calculations for the bioaccumulation (BAF) and biotransfer factor (BTF). From these results it can be concluded that the bioaccumulation factor (transfer of the element from soil to all parts of the plant) was very low for As (0.023 in *Onobrychis degenii* and 0.073 in *Knautia caroli-rechingeri*, while for *Centaurea kavadarensis* it could not be calculated because the contents of As in the parts of the plants were below the detection limit) and for Sb. From the other side, BAF for thallium was higher ranging from 0.322 in *Onobrychis degenii* and 0.618 in *Knautia caroli-rechingeri*, and the highest values of 3.819 were found for *Centaurea kavadarensis*.

Biological transfer factor (BTF), which represents the ratio between the content of that element in the flower (or seeds) and in the root, was the highest also for Tl (from 0.048 in *Knautia caroli-rechingeri* to 0.737 in *Centaurea kavadarensis*) which shows their capability to accumulate high amounts of Tl. Probably this behavior of thallium could be explained by the interpretation given by Madejon et al. (2007) that when Tl was present in soils it may be easily taken up by plants because it generally presents as thermodynamically stable Tl(I), an analogue to potassium, or by the explanation that thallium(I) has a very low stability that is constant with both organic and inorganic ligands and therefore should be easily extractable from the leaves (Kaplan and Mattigod 1998; Nriagu 1998; Scheckel et al. 2004; Bačeva et al. 2014b).

13.4 Conclusion

The aim of this study was to increase the knowledge about the behavior of a wide range of elements with special attention paid to As, Sb, and Tl in seven local endemic plant species: *Viola allchariensis* G. Beck, *Viola arsenica* G. Beck, *Thymus alsarensis* Ronn., *Centaurea leucomalla* Bornm., *Onobrychis degenii* Dörfler, *Knautia caroli-rechingeri*, and *Centaurea kavadarensis,* and one subendemic species – *Viola macedonica* Boiss. and Heldr., in the environment of the As-Sb-Tl mining area where they are exposed to large amounts of these elements present in the soil (the locality of Allchar, Republic of North Macedonia).

The data showed that *Viola* species are able to hyperaccumulate toxic elements in their roots, stems, leaves, flower, and seeds. The content of As and Sb was significantly higher in the roots and seeds, while the content of Tl was significantly higher in the leaves, flowers, and seeds in all *Viola* species. Data from the extractions for plant-available elements of soil samples performed in water, HCl, and buffered DTPA solutions show that As was the most extractable from HCl solution and the extractability of Tl was similar for all *Viola* species, while the extractability of Sb was much lower than that of As and Tl.

The content of As was higher in the roots, leaves, and stems of *Thymus* species, while the content of Tl was significantly higher in the roots, flowers, and leaves in all investigated *T. alsarensis* specimens. Such behavior of As, Sb, and Tl was confirmed by extraction tests with various solvents of soil samples collected from the same locations as *Thymus* specimens. It was established that in the extraction solutions of 0.1 M HCl, water, or DTPA, the mostly available element was Tl.

The content of As was significantly higher in the roots, leaves, and stems, while the content of Tl was significantly higher in the roots of *Centaurea* specimens which was also confirmed by the extraction tests with various solvents of soil samples collected from the same locations. It was established that in all extraction solutions, Tl was the most extractable element.

The results showed that the accumulation of As and Tl in other endemic species from the Allchar locality (*Onobrychis degenii*, *Knautia caroli-rechingeri*, and *Centaurea kavadarensis* species) was significantly high, and they were able to hyperaccumulate toxic elements in their roots and shoots. Accumulation of these elements in the studied endemic species may be used as a valuable tool for bioindication, while, on the other hand, the accumulation of these toxic elements in the plants causes high risk to human and animal health. It was established that in all extraction solutions, Tl was the most extractable element.

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Index

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